



**LIBRARY**  
New Delhi

Call No

Acc No

**25033**

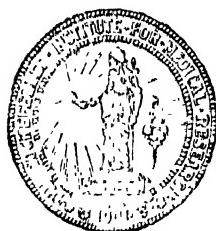




STUDIES  
FROM  
THE ROCKEFELLER INSTITUTE  
FOR MEDICAL RESEARCH

REPRINTS

VOLUME XXXV



NEW YORK  
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH  
1921

*Results of the investigations conducted at The Rockefeller Institute, or elsewhere under its grants, are first reported in a variety of journals and publications. The reports are then assembled in volumes designated Studies from The Rockefeller Institute for Medical Research, of which this is Number XXXVI. The Studies appear serially but at irregular intervals. The text of the original publications is in all respects followed in the Studies. The name, date, number, and pages of the journal in which each article originally appeared are printed above the title. To insure uniformity and simplicity of reference, plates and illustrations repeat the numbers used in the place of first publication.*

COPYRIGHT, 1921, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS  
THE WILLIAMS & WILKINS COMPANY  
BALTIMORE, U. S. A.

# CONTENTS.

---

## PATHOLOGY AND BACTERIOLOGY.

	PAGE
MURPHY, JAMES B., and NAKAHARA, WARO. The lymphocyte in natural and induced resistance to transplanted cancer. V. Histological study of the lymphoid tissue of mice with induced immunity to transplanted cancer.....	1
NAKAHARA, WARO, and MURPHY, JAMES B. Studies on x-ray effects. V. Effect of small doses of x-rays of low penetration on the lymphoid tissue of mice.....	13
OLITSKY, PETER K., and KLIGLER, I. J. Toxins and antitoxins of <i>Bacillus dysenteriae</i> Shiga.....	19
GATES, FREDERICK L. A method of standardizing bacterial suspensions.....	35
FLEXNER, SIMON, and AMOSS, HAROLD L. Experiments on the nasal route of infection in poliomyelitis.....	45
NOGUCHI, HIDEYO. Etiology of yellow fever. X. Comparative immunological studies on <i>Leptospira icteroides</i> and <i>Leptospira icterohæmorrhagiae</i> .....	57
NOGUCHI, HIDEYO. Etiology of yellow fever. XI. Serum treatment of animals infected with <i>Leptospira icteroides</i> .....	81
LAWSON, MARY R. Crescentic bodies in æstivo-autumnal malaria; their migration and attachment to the surface of the red corpuscle.....	91
ROUS, PEYTON, WILSON, GEORGE W., and OLIVER, JEAN. Experiments on the production of specific antisera for infections of unknown cause. III. The effects of a serum precipitin on animals of the species furnishing the precipitinogen.	99
UHLENHUTH, EDUARD. Regeneration and neoteny.....	113
FLEXNER, SIMON. Lethargic encephalitis. History, pathologic and clinical features, and epidemiology in brief.....	119
BROWN, WADE H., and PEARCE, LOUISE. Experimental syphilis in the rabbit. I. Primary infection in the testicle.....	133

- ROUS, PEYTON, and LARIMORE, LOUISE D. Relation of the portal blood to liver maintenance. A demonstration of liver atrophy conditional on compensation..... 157

## CHEMISTRY.

- LEVENE, P. A. Crystalline uridinphosphoric acid..... 181  
 LEVENE, P. A. The structure of yeast nucleic acid. V.  
     Ammonia hydrolysis..... 183  
 LEVENE, P. A. Properties of the nucleotides obtained from yeast nucleic acid..... 189

## EXPERIMENTAL BIOLOGY.

- LOEB, JACQUES. Influence of a slight modification of the collodion membrane on the sign of the electrification of water.. 201  
 LOEB, JACQUES. Influence of the concentration of electrolytes on some physical properties of colloids and of crystalloids... 219  
 LOEB, JACQUES. Quantitative laws in regeneration. I..... 243  
 NORTHROP, JOHN H. A device for regulating the temperature of incubators either above or below room temperature..... 255  
 NORTHROP, JOHN H. Concerning the hereditary adaptation of organisms to higher temperature..... 259  
 LOEB, JACQUES. The nature of the directive influence of gravity on the arrangement of organs in regeneration..... 265  
 LOEB, JACQUES. On the cause of the influence of ions on the rate of diffusion of water through collodion membranes. I. 279

## THE HOSPITAL OF THE ROCKEFELLER INSTITUTE.

- STADIE, WILLIAM C., and VAN SLYKE, DONALD D. Studies of acidosis. XV. Carbon dioxide content and capacity in arterial and venous blood plasma..... 301  
 STADIE, WILLIAM C. A method for the determination of methemoglobin in blood..... 307  
 LEVY, ROBERT L., and CULLEN, GLENN E. Deterioration of crystalline strophanthin in aqueous solution. Its relation to hydrogen ion concentration and a method for its prevention..... 313

AUSTIN, J. HAROLD, and VAN SLYKE, DONALD D. Determination of chlorides in whole blood .....	321
HOLT, L. EMMETT, COURTNEY, ANGELIA M., and FALES, HELEN L. Calcium metabolism of infants and young children, and the relation of calcium to fat excretion in the stools. Part I. Infants taking modifications of cow's milk.....	325
HOLT, L. EMMETT, COURTNEY, ANGELIA M., and FALES, HELEN L. Calcium metabolism of infants and young children, and the relation of calcium to fat excretion in the stools. Part II. Children taking a mixed diet.....	347
VAN SLYKE, DONALD D., and PALMER, WALTER W. Studies of acidosis. XVI. The titration of organic acids in urine...	377
CULLEN, GLENN E., and VAN SLYKE, DONALD D. Determination of the fibrin, globulin, and albumin nitrogen of blood plasma.....	397
ALLEN, FREDERICK M. Experimental studies on diabetes. Series I. Production and control of diabetes in the dog.	
1. Gross anatomic relations of the pancreas and diabetes ...	409
ALLEN, FREDERICK M. Experimental studies on diabetes. Series I. Production and control of diabetes in the dog.	
2. Effects of carbohydrate diets.....	427
ALLEN, FREDERICK M. Experimental studies on diabetes. Series I. Production and control of diabetes in the dog.	
3. Effects of protein diets.....	449
ALLEN, FREDERICK M. Experimental studies on diabetes. Series I. Production and control of diabetes in the dog.	
4. Control of experimental diabetes by fasting and total dietary restriction.....	469
ALLEN, FREDERICK M. Experimental studies on diabetes. Series I. Production and control of diabetes in the dog.	
5. Various failures of dietetic treatment, and their causes... 481	

## ANIMAL PATHOLOGY.

BROWN, J. HOWARD. The cultural differentiation of beta hemolytic streptococci of human and bovine origin.....	503
BROWN, J. HOWARD, and ORCUTT, MARION L. Dairy infection with <i>Streptococcus epidemicus</i> .....	517

SMITH, THEOBALD. Mycosis of the bovine fetal membranes due to a mould of the genus <i>Mucor</i> .....	539
JONES, F. S. Source and significance of streptococci in market milk .....	547
SMITH, THEOBALD, and GRAYBILL, H. W. Epidemiology of blackhead in turkeys under approximately natural conditions .....	563
GRAYBILL, H. W., and SMITH, THEOBALD. Production of fatal blackhead in turkeys by feeding embryonated eggs of <i>Heterakis papillosa</i> .....	577

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, January 1, 1920, Vol. xxxi,  
No. 1, pp. 1-11.]

## THE LYMPHOCYTE IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

### V. HISTOLOGICAL STUDY OF THE LYMPHOID TISSUE OF MICE WITH INDUCED IMMUNITY TO TRANSPLANTED CANCER.

By JAMES B. MURPHY, M.D., AND WARO NAKAHARA, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 1 AND 2.

(Received for publication, October 29, 1919.)

A number of theories have been brought forward to explain the natural and induced resistance to the transplanted cancers of mice, but none of them has covered all the facts or met with general acceptance.

The so called athrepsia theory of Ehrlich<sup>1</sup> has been the most prominent of these explanations, but in the light of more recent work it apparently does not account for the phenomenon. This theory was based on the observation that mouse tumors inoculated into rats or *vice versa* were capable of survival and growth for a time but would die if allowed to remain. If, however, the graft was removed from the rat during the proliferative stage and then returned to the mouse it would continue to grow and after a period could again be transplanted to rats. This zigzag grafting could be continued indefinitely. The interpretation offered by Ehrlich was that each species produces a specific and limited amount of food substance necessary for growth (substance X). The temporary survival and growth of the mouse tumor in the rat was accounted for by the amount of substance X carried over with the graft, and when this was exhausted the mouse tissue died from lack of food. When the tumor was returned to the mouse it would accumulate a fresh supply of the substance and would be able again to live for a time in the rat. Lambert and Hanes<sup>2</sup> showed that a tissue of one species could live and grow for some time in the plasma of another. In fact, growth was almost as good as when the tissues were placed in homologous plasma. Murphy<sup>3</sup> demonstrated that mammalian tissue could be carried through

<sup>1</sup> Ehrlich, P., *Arb. k. Inst. exp. Therap. Frankfurt*, 1906, No. 1, 84.

<sup>2</sup> Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiv, 129.

<sup>3</sup> Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

many generations in the chick embryo without return to its native species. Furthermore, he showed that rats, when subjected to suitable exposures of x-rays, were capable of supporting a mouse tumor in active growth over prolonged periods with repeated transplantation.<sup>4</sup> From the facts of these investigations the atresia theory does not seem to meet the conditions.

A theory, brought forward by Russell<sup>5</sup> and strongly supported by the workers of the Imperial Cancer Research Fund, attributes cancer resistance to a failure of the stroma reaction. Thus, a graft in a resistant animal fails for lack of suitable blood supply and framework to support growth. This explanation has not met with wide acceptance, for it does not explain naturally acquired resistance. Moreover, a number of observers have described failure of cancer grafts in resistant animals in which there is an abundant vascular and stroma reaction (Burgess,<sup>6</sup> Rous,<sup>7</sup> Goldmann,<sup>8</sup> Levin<sup>9</sup>).

Tyzzer,<sup>10</sup> finding an identical histological reaction in resistant animals, whether the immunity was induced or natural, concludes that the same factors are responsible for both conditions. The chief difference is the time at which the reaction takes place, which is earlier in the animals with induced resistance than in those with natural resistance. He expresses the idea that the associated "inflammation" is the defensive factor. The reaction is characterized in the early stages by an accumulation of polymorphonuclear leucocytes around the graft, followed later by lymphocytic infiltration and an increase in the connective tissue elements. The first stage, the polymorphonuclear accumulation, is the same in the susceptible and resistant animals and so may be disregarded in a consideration of the immunity factors. Tyzzer suggests that the failure of the graft in these cases is due to a choking of the blood supply and a starvation of the introduced cells. The view we have favored is that the immunity is due to cellular reaction but that the lymphoid elements are the important agents in this process.

Da Fano<sup>11</sup> was the first to call attention to the lymphocyte as the possible active agent in cancer immunity. He noted the fact that there was not only an accumulation about the graft in resistant animals but also an increase in the numbers of these cells in the subcutaneous tissues. Baeslack<sup>12</sup> observed a percentage increase in the circulating lymphocytes in a very small series of immune

<sup>4</sup> Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

<sup>5</sup> Russell, B. R. G., in Bashford, E. F., *3rd Sc. Rep., Imperial Cancer Research Fund*, 1908, 341.

<sup>6</sup> Burgess, A. M., *J. Med. Research*, 1909, xxi, 575.

<sup>7</sup> Rous, F. P., *J. Am. Med. Assn.*, 1910, lv, 1805.

<sup>8</sup> Goldmann, E. E., *Beitr. klin. Chir.*, 1911, lxxii, 1.

<sup>9</sup> Levin, I., *J. Expt. Med.*, 1911, xiii, 604; xiv, 139.

<sup>10</sup> Tyzzer, E. E., *J. Cancer Research*, 1916, i, 125.

<sup>11</sup> Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

<sup>12</sup> Baeslack, F. W., *Z. Immunitätsforsch., Orig.*, 1913-14, xx, 421.

animals. Murphy and Morton<sup>13</sup> first showed that there was an actual increase in the circulating lymphocytes in animals during the development of active resistance, both when induced and when naturally acquired. The workers in this laboratory have extended this work to show a close relation between the lymphocyte reaction and the resistance to cancer. Mice rendered potentially immune by the injection of blood can be made susceptible by the destruction of the lymphoid tissue.<sup>13</sup> Likewise, mice of tested immunity, after suitable exposure to x-rays, become susceptible to inoculation.<sup>14</sup> These observations of Murphy and his coworkers have been confirmed and extended by Mottram and Russ.<sup>15</sup> Moreover, the former found that animals with an artificially induced lymphocytosis<sup>16</sup> become as highly resistant to cancer implants as they do following tissue injection.<sup>17</sup> In the latter experiment a parallelism was noted between the number of lymphocytes in the blood and the number of mitotic figures in the spleen and lymph glands.<sup>18</sup>

In the work presented in this paper a study has been made of the lymphoid organs in animals with induced immunity to cancer to establish a further link in the evidence associating the lymphocyte with cancer immunity and to ascertain if possible the source and nature of the blood lymphocytosis. In the course of the experiments a histological examination was made of the changes in the subcutaneous tissue in order to check and possibly extend the earlier observation of Da Fano.<sup>11</sup>

#### *Material.*

The material for the study has been collected in the course of five experiments embracing over 100 mice, as described in the following protocols.

*Experiment 1.*—Thirty-four white mice were given an injection of 0.3 cc. of defibrinated mouse blood in the subcutaneous tissue of the back. Three mice were killed on the 6th day and three on the 10th day after the immunizing in-

---

<sup>13</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

<sup>14</sup> Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

<sup>15</sup> Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917, xc, 1.

<sup>16</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

<sup>17</sup> Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25. Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

<sup>18</sup> Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17, 83.

jection. The remaining twenty-eight mice were inoculated, 10 days after the blood injection, with bits of Bashford Adenocarcinoma No. 63. They were killed for histological examination in groups, 24 hours, and 5, 15, 25, and 35 days after the inoculation.

*Experiment 2.*—Twenty-seven white mice were immunized with a subcutaneous injection of defibrinated blood as in Experiment 1. They were killed in groups 24 and 48 hours, and 4, 6, 8, and 10 days after the treatment and sections taken of the lymphoid organs.

*Experiment 3.*—Ten mice were immunized as in the previous experiments and 10 days later were inoculated with bits of Bashford Adenocarcinoma No. 63 as in Experiment 1. They were killed in groups and tissues taken at daily intervals from 24 hours to 4 days after the inoculation.

*Experiment 4.*—Twenty-eight normal mice were immunized as before. Four mice were killed for tissues 4 days afterwards, and on the 10th day after immunization four more were killed. The remaining twenty immunized mice were inoculated with bits of Bashford Tumor No. 63 10 days after the immunizing injection. They were killed in groups for tissues 2, 5, 15, 25, and 35 days after the inoculation.

*Experiment 5.*—Nine normal white mice were inoculated intraperitoneally with 0.6 cc. of defibrinated blood from normal mice. These were killed, three at a time, 24 hours, and 3 and 5 days after the inoculation, and the subcutaneous connective tissue was taken for examination.

All the mice used in the experiments were of about the same size and were from the same stock. The virulence of the tumors used in each experiment was tested by inoculation into a number of normal mice.

The fixative used for tissues was Conroy's 6-3-1, and the staining was done with Heidenhain's iron-hematoxylin for mitotic figures and eosin-methylene blue for other general purposes. In most instances loose connective tissue from the subcutaneous layer was carefully spread over the slide, fixed with absolute alcohol, and stained with methylene blue and eosin. Blood films were treated with Wright's stain.

#### OBSERVATIONS.

Much variation has been noted in the extent of the reaction in different animals. It has been shown by experiment that a certain percentage of mice are not immune after tissue injection but will grow tumors actively. In the earlier stages of the immunity reaction at which we have studied the lymphoid tissue it is unfortunately im-

possible to predict which of the animals would have fallen into the non-immune group. Therefore we are unable to say whether the animals failing to show the typical reaction of the majority would have proved to be non-immune if they had been allowed to live.

*Spleen.*—The stimulation of germinal centers was manifest 48 hours after the blood injection. In a section taken at this stage an average nodule usually contained a few well marked mitotic figures, three to five as a rule, more rarely six or seven. All stages of mitosis were easily distinguished. Among the cells of the germinal center were found a few large cells with pycnotic and fragmented nuclei. There was the usual number of pigmented cells and megalocaryocytes in the pulp spaces. The frequency of mitosis in the germinal center after 4 days was apparently greater than before (Fig. 1). There was also a slight increase in the number of pigmented cells and megalocaryocytes. After 5 days the number of mitotic figures was seen to decrease somewhat. The abundance of megalocaryocytes and pigmented cells at this time was about the same as before. There were small groups of pycnotic cells appearing in the pulp at about this period. Similar conditions seemed to continue until about 10 days, when the proliferative activity of germinal centers returned nearly to the normal level, but a few mitotic figures were seen at this period also. Moderate numbers of pycnotic cells were also found in the nodules.

The cytological condition just described was more strongly marked 24 to 48 hours after the cancer inoculation (Fig. 2). At this period there was a general enlargement of the splenic nodules and the latter contained numerous mitotic figures. The change was far more extensive than that occurring in the previous period. The number of pycnotic cells in the nodules was insignificant.

The enhanced cell division was conspicuous for about 1 week following the cancer inoculation, but after this period the normal rate was gradually resumed. Toward the later period large numbers of pycnotic cells were found in the nodules, but pigmented cells were always infrequent.

About 35 days after the inoculation the general histological appearance of the organ had returned approximately to normal.

*Lymph Glands.*—The cytological changes observed in the mesenteric and inguinal lymph glands were very similar to those found in the spleen. A distinct acceleration in the rate of cell division in the lymph glands was observed 24 hours after the immunizing injection. Dividing cells were numerous in the germinal centers of the nodules and were not infrequent even in the lymph cord. At 48 hours the number of dividing cells was more or less decreased, but there was considerable variation among individual mice. Slight stimulation was indicated by the unusual frequency of mitosis as late as 10 days after the treatment. No appreciable change was observed in the medulla.

The germinal center of the nodule became decidedly restimulated soon after the cancer inoculation, as evidenced by numerous mitotic figures, and this condition, in a less marked degree, lasted for a considerable length of time. About 35 days after the inoculation, or probably a little earlier, cell division in the nodule subsided to the normal rate.

No special change has been observed in any other part of the gland.

*Circulating Lymphocytes.*—Lymphocytes in the circulating blood of immunized animals were often seen in the process of amitosis, especially during the first several days after the inoculation of cancer. Amitosis was observed in large as well as small lymphocytes, but more frequently in the former. Cells in typical stages of amitosis were found among others showing irregularly shaped, lobulated nuclei.

The accepted interpretation of amitosis is that it is not a method of cell multiplication<sup>19</sup> but a means of increasing the nuclear surface to meet intensified metabolic activities of the cell.<sup>20</sup> Since the lymphocytes are presumably in their active functioning stage at the period just indicated, this conception may, in the present state of our knowledge, be applied to the case under consideration. Even if two caryomeres produced by amitosis eventually separate into two apparent cells, we would not be warranted at present in considering the process evidence of genuine cell multiplication, especially in view of the scanty amount of cytoplasm possessed by the resulting cells.

<sup>19</sup> Conklin, E. G., *Biol. Bull.*, 1917, xxxiii, 396.

<sup>20</sup> Nakahara, W., *J. Morphol.*, 1917-18, xxx, 483.

*Subcutaneous Connective Tissue.*—As early as 24 hours after the injection of the blood a well marked cellular reaction was seen in the connective tissue about the groups of red cells. The great majority of cells participating in this reaction were small lymphocytes. Other types of cells such as polymorphonuclear leucocytes and macrophages were rare. After about 4 days, when the injected blood became more generally distributed in the subcutaneous connective tissue, the local reaction became more diffuse. Now, besides the large number of lymphocytes, plasma cells in considerable number (Fig. 3) were present, and macrophages, which were few in earlier periods, became abundant. Owing to the wide distribution at this period of the injected red cells, it was found that an extensive area of the subcutaneous tissue showed numerous lymphocytes and other white cells. Soon the area infiltrated by red cells diminished, and there was a corresponding lessening of the lymphoid reaction. This cellular infiltration continued for about 8 days, after which time only slight traces of blood could be recognized.

The cellular reaction about the cancer graft in naturally resistant or artificially immunized animals bears a close similarity to the reaction as described above. As has been shown by many previous investigators, an extensive inflammatory reaction, which is lymphoid in nature, precedes the destruction of the cancer tissue, but the reaction around the graft subsides promptly after the latter becomes completely necrotic.

The cells which participate in this reaction are in both instances mainly lymphoid. Da Fano's<sup>11</sup> cellular analysis of the inflammatory reaction about the regressive cancer shows that the lymphocyte, plasma cell, and macrophage are characteristic of the reaction. Since these cells are usually absent in normal subcutaneous tissue of the mouse, much significance has been attached to their abundance about the neoplasm.

In addition to the local reaction about cancer tissue, Da Fano claims that the plasma cells which are absent in normal connective tissue of the mouse appear 48 hours after the injection of the blood for immunization and they become more numerous in the succeeding days until the 4th day, when small groups of the cells are seen in every section. While Da Fano's statements were confirmed in some of our

experiments, in others we failed to confirm them. We found no lymphoid proliferation in the loose connective tissue at a distance from the area infiltrated with red blood cells, and the loose connective tissue was found to be apparently normal, even when local lymphocytic infiltration was well pronounced about the injected blood cells or cancer graft (compare Figs. 3 and 4). That Da Fano has misinterpreted the local reaction about the mass of injected blood cells as a general reaction in immunity becomes clear when the immunizing dose is given intraperitoneally. In the latter case although the immunity is effective, no appreciable lymphoid proliferation in the loose connective tissue results.

*Other Organs.*—The thymus and thyroid glands, the liver, kidney, and bone marrow were studied, but no special changes were detected. In a few instances there was a rather unusually large number of mitotic figures in the cells of the thymus, and mitotic figures were often seen among the lymphoid cells about the vessels of the liver. But these active cells were either too few in number or too inconstant in occurrence to be more than mentioned in connection with the immunity reaction.

#### DISCUSSION.

The conclusions suggested by the work of Murphy and Morton regarding the rôle of the lymphocyte in immunity to transplanted cancer are not only in harmony with previous observations on the factor of resistance to heteroplastic tissue grafting and on cellular reactions about the cancer graft in animals with natural or induced immunity, but have sustained subsequent experimental tests as well. An objection which may be raised against the original work is that the evidence of lymphoid proliferation was based entirely upon the results of blood cell counts. Tyzzer,<sup>10</sup> for instance, appreciating the wide range of variability in the white cell counts of the tail vein in normal mice, expresses the opinion that the increase in the number of lymphocytes in the blood of immune mice indicated by Murphy and Morton may probably be greatly exaggerated. The validity of this objection has now been set aside by the present cytological work, which shows that, accompanying the lymphocytosis, there is a corresponding enhancement of the rate of cell division in lymphoid centers.

In the same article Tyzzer points out, as of considerable significance, the fact that no change in the lymphocyte count of the mice is seen after the immunizing injection, while, as a matter of fact, "an animal so treated now possesses qualities which were previously absent, or in other words had become immune." In the light of the facts brought out in the present paper this point may simply mean that the newly formed lymphocytes do not get into circulation in sufficient number to change the general blood picture until the inoculation of cancer is made. It is now clear that an animal which has become potentially immune possesses hypertrophic lymphoid elements, although this fact is not reflected in the blood counts. However, the mechanism of the lymphoid reaction has become, as it were, sensitized so that a very small amount of tumor is sufficient to induce a relatively large blood lymphoid response.

Another finding of special interest is the similarity of the cellular reaction about the injected blood to that about the cancer graft in the immunized animal. The types of cells which participate in the reaction are in both instances primarily lymphoid, including large and small lymphocytes, plasma cells, and phagocytic cells. Moreover, the two reactions are to be regarded as of the same nature in that they no longer continue after the invading cells are dead. This point becomes more significant when taken together with the fact that the cells in the lymphoid centers respond in the same way to the blood injection as they do to the cancer inoculation, the only difference being one of degree. It seems probable, therefore, that the lymphoid cells of an untreated animal are sufficient in quantity and quality to be an efficient defensive mechanism against such cells as red blood cells, with no proliferative ability, and against such other tissues as have been used to produce immunity, such as spleen, embryonic tissue, skin, etc. As a result of the primary stimulus the capacity of the lymphoid tissue to react has become so enhanced that it is capable of a massive reaction when the cancer is inoculated.

The irregularity of the results described in our present experiments were to be expected. It is well known that a proportion of mice immunized to cancer show no more resistance than normal mice, while in still others the resistance to cancer growth is at first not evident, the defensive mechanism asserting itself sufficiently to over-

come the cancer only after a period of growth has occurred. Unfortunately at the time of the greatest changes in the spleen and lymph glands it is impossible to predict which way the animals would have arranged themselves according to this grouping. To take an example, ten mice are inoculated with cancer fragments 10 days after receiving an immunizing injection. If they are allowed to live the result would be as follows, judging by the average experiment with our strain of tumor: about two would develop tumors, about two more would show a temporary growth followed by retrogression, and the remaining six would be immune from the beginning. If all these animals are killed during the first few days after inoculation we have no way of comparing the extent of the reaction in the spleen with the degree of immunity which the animal might have shown if allowed to live. Therefore we would expect, if the changes in the spleen and lymph glands are an index to the immunity, only a proportion of the mice to show a marked reaction and from 10 to 20 per cent a delayed reaction. In our previous studies of the blood it was noted that the degree of immunity followed fairly closely the extent of the lymphocytosis.

#### SUMMARY AND CONCLUSIONS.

Mice immunized against cancer by means of an injection of defibrinated blood show in the germinal centers of the lymphoid organs a marked increase in the numbers of mitotic figures. The increase becomes evident 48 hours after the injection in the majority of instances and reaches its climax at about the 5th day. After this time it subsides, returning to the normal rate about the 10th day.

These immunized animals, when inoculated with a cancer graft 10 days after the injection, show a second stimulation of the lymphoid centers similar to the first but more intense in character. This increase in the number of mitotic figures becomes evident as early as 24 hours after the cancer inoculation and persists in a marked degree for a week, after which there is a gradual return to the normal rate.

The lymphocytes of the circulating blood during the establishment of the immunity show frequent examples of amitotic division, and many examples of irregular and lobulated nuclei. These changes suggest intensified functional activity.

Contrary to the statement of Da Fano, cellular reaction in the subcutaneous tissues of immunized animals is present only in the region infiltrated by the injected cells. This fact becomes conspicuous when the immunizing injection is given intraperitoneally, in which case no cellular accumulations are noted in the loose connective tissues.

No constant cellular changes were noted in the bone marrow, thymus or thyroid gland, liver, or kidney of the treated animals.

#### EXPLANATION OF PLATES.

##### PLATE 1.

FIG. 1. Germinal center of the spleen 4 days after the blood injection. *M*, mitotic figure.

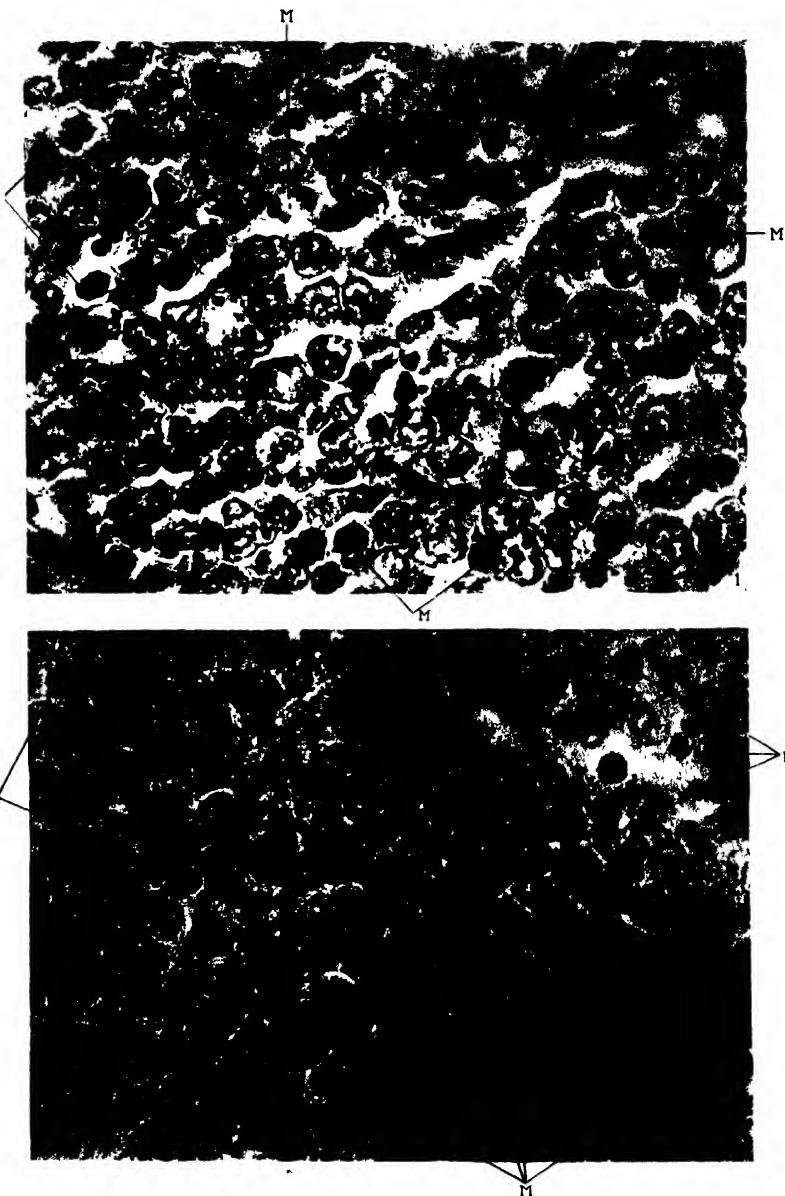
FIG. 2. The same, 48 hours after the cancer inoculation in the immunized mouse. *M*, mitotic figure.

##### PLATE 2.

FIG. 3. Lymphoid infiltration in the subcutaneous tissue of an immunized mouse, the dark background indicating the presence of injected blood.

FIG. 4. A portion of subcutaneous tissue of an immunized mouse free from the injected blood, showing apparently normal cellular conditions.

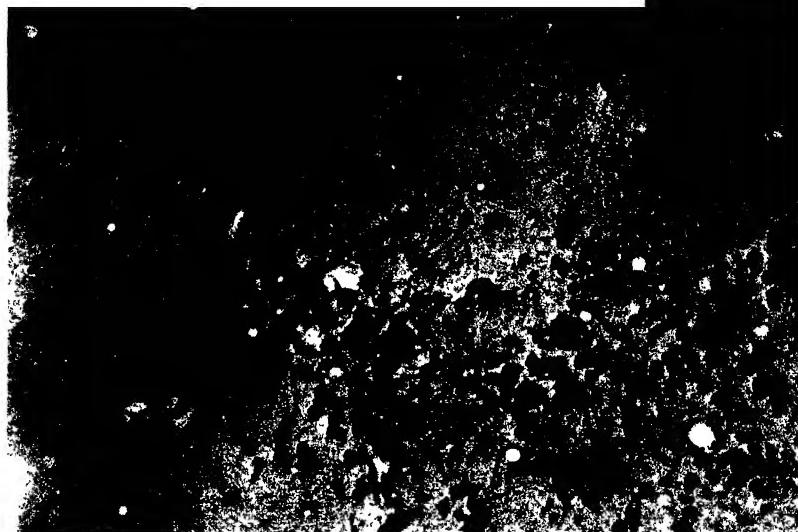




(Murphy and Nakahara: Resistance to transplanted cancer. V.)



THE JOURNAL OF EXPERIMENTAL MEDICINE VOL. XXXI.



(Murphy and Nakahara: Resistance to transplanted cancer. V.)



[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, January 1, 1920, Vol. xxxi,  
No. 1, pp. 13-17.]

## STUDIES ON X-RAY EFFECTS.

### V. EFFECT OF SMALL DOSES OF X-RAYS OF LOW PENETRATION ON THE LYMPHOID TISSUE OF MICE.

BY WARO NAKAHARA, PH.D., AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 3 TO 5.

(Received for publication, October 29, 1919.)

The destructive effect of x-rays on the lymphoid tissue was early noted in the study of the biological effects of this agent. The stimulating action of this agent on the circulating lymphocytes was first observed in this laboratory and was applied experimentally in the study of x-ray effects on spontaneous tumors of mice.<sup>1</sup> The earlier work was carried out with the old type of gas tube, with which it was difficult to regulate the amount and character of rays used, and therefore no attempt was made to standardize the dosage. This difficulty has largely been overcome by the use of the Coolidge tube. The stimulating dose for rabbits has been reported.<sup>2</sup> The work of Russ, Chambers, Scott, and Mottram<sup>3</sup> confirms our earlier observation on the stimulative action in mice.

In our experiments with rabbits a histological study paralleling the blood counts confirmed the general nature of the stimulation by showing a marked increase in the number of mitotic figures in the germinal centers of the lymphoid organs of these animals.<sup>4</sup> As mice are the animals used most extensively in our experiments, it was regarded as important to duplicate the histological study of the

<sup>1</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

<sup>2</sup> Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

<sup>3</sup> Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919, i, 692.

<sup>4</sup> Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83

lymphoid organs of the latter animals after a stimulating dose of x-rays. With this end in view, the following experiments were undertaken.

### *Methods.*

In the analysis of the artificial stimulation of the lymphocytes the reaction in the lymphoid germinal centers must be studied first. The spleens and lymph glands from the stimulated animals were fixed with Conroy's 6-3-1 fluid and stained with Heidenhain's iron-hematoxylin. The reaction was analyzed mainly from the standpoint of the frequency of mitosis.

### EXPERIMENTS.

*Experiment 1.*—Seven normal white mice were placed in a glass jar and exposed to the following dose of x-rays: spark-gap  $\frac{1}{4}$  inch between points, milliamperage 25, distance from the target to the back of the animal approximately 8 inches, time 20 minutes. The top of the jar was covered to shield the animal from the heat of the tube. The mice were killed 24 hours, and 3, 5, 7, 10, 12, and 14 days, respectively, after the treatment and the lymphoid organs examined histologically.

24 hours after treatment numerous degenerated cells, with pycnotic or fragmented nuclei appeared in the spleen (Fig. 1). The Malpighian bodies were small and inconspicuous. The general histological condition simulated somewhat the picture described by Heineke<sup>5</sup> and Warthin<sup>6</sup> after a massive dose of x-rays, but the changes were not so extensive. No appreciable amount of pigment was seen, but there was a moderate number of mitotic figures distributed irregularly. The necrotic changes were not so marked in the lymph glands as in the spleen, but mitotic figures were almost totally absent from the former.

3 days after treatment the necrotic cells were found to be decreased in number. The Malpighian bodies of the spleen were more or less evident but showed few mitotic figures.

After 5 days, and up to 14 days, the general histological condition of the lymphoid organs was about normal. There was no increase in the number of mitotic figures.

<sup>5</sup> Heineke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

<sup>6</sup> Warthin, A. S.; *Physician and Surg.*, 1907, xxix, 1.

It must be concluded that no lymphoid stimulation occurred in the mice used in the preceding experiment. The dose of x-rays used was one which yielded evidences of lymphoid stimulation without appreciable destruction in rabbits.

*Experiment 2.*—Three normal white mice were treated in the same way and with the same dose of x-rays as the mice in Experiment 1, except that the time of exposure was reduced to 10 minutes. These animals were killed 24 hours, and 4 and 8 days, respectively, after the treatment and the lymphoid organs studied.

A considerable increase in the number of mitotic figures in the nodule of the spleen (Fig. 2) was seen at 24 hours and at 4 days after treatment with x-rays. The rough estimate of the average number of these figures in a section of one nodule of the spleen was five or six at these periods. This is in striking contrast with the normal condition, in which mitotic figures in a splenic nodule are few. No other special change was observed, except an appreciable increase of pycnotic cells in the pulp at 24 hours after x-rays. This, however, was not so conspicuous as in the previous experiment.

The lymph glands showed an equally pronounced increase in the mitotic figures at the corresponding period (Fig. 3), and figures were also seen frequently in the medulla.

8 days after the x-ray treatment the spleen and lymph glands, as regards the number of mitotic figures, were normal in appearance, but gave the impression of having more abundant lymphoid elements.

*Experiment 3.*—Five normal white mice were x-rayed as before. In this experiment, however, the time of exposure was reduced to 5 minutes, the other factors remaining unchanged. The mice were killed 24 hours, and 4, 7, 10, and 14 days, respectively, after the exposure.

Histological examination of the lymphoid organs showed no marked change, as regards the number of mitotic figures, throughout the periods studied. Moreover, the evidences of destruction which were so marked in the first experiment and much less so in the second one were not present.

From the three series of experiments described we may say that the indication is that stimulation of lymphoid tissue in the mouse may be effected by a 10 minute exposure to a small dose of x-rays. To

establish this point the following additional experiments were made, triplicating Experiment 2.

*Experiment 4.*—Three normal mice were exposed to x-rays of the same quality as in the previous experiments for 10 minutes. They were killed 24 hours, and 4 and 8 days, respectively, after the treatment.

*Experiment 5.*—Five normal mice were treated with the 10 minute dose as before and were killed 24 hours, and 3, 5, 7, and 10 days, respectively, after treatment.

In the mice killed at 24 hours and 4 days after treatment in Experiment 4 and in those killed 24 hours and 3 days after treatment in Experiment 5, an abnormally large number of mitotic figures was observed in the lymphoid centers, just as had been observed in Experiment 2. The other mice of this series showed no such change. The initial destruction was the same as that observed in Experiment 2.

#### DISCUSSION.

The uniformity of the changes, as shown in the three experiments, both in extent and period of occurrence, cannot be considered as a mere coincidence. It is to be concluded, therefore, that the small dose of x-rays employed in these experiments is capable of stimulating to proliferation the lymphoid tissue of mice.

It is of interest to note in this connection the apparent relation between the extent of cellular destruction and the degree of cellular stimulation, as shown in Table I. It would seem from these observations that a certain amount of destruction is followed by proliferation, which occurs very slightly, if at all, after too much or too little destruction. While no definite conclusion should be drawn on this point from our small series of experiments, they seem to be of sufficient interest to be reported.

TABLE I.

Time of exposure.	Destruction.	Stimulation.
min.		
20	+	±
10	±	++
5	-	-

From our present knowledge of the subject it is safe to say that the quantitative increase of the lymphoid elements in the body is mainly due to the hyperactivity of the lymphoblastic tissue of the lymphoid organs. Because of this activity hypertrophy of lymphoid organs, especially of the Malpighian bodies, and lymphocytosis in the blood may both result. Theoretically, as regards the number of these cells thrown into the circulation, it is conceivable that individual animals may react differently even with an equal stimulation of the lymphoid organs. Cases have been observed in which the blood lymphocytosis was due apparently to the mere emptying of the lymphoid organs without a corresponding actual increase of the lymphoid cells having taken place; and the histological studies just described have certainly given more nearly uniform evidence of stimulation than have blood counts.

#### CONCLUSION.

A dose of x-rays governed by the following factors induces a stimulation of lymphoid tissue in mice: spark-gap  $\frac{1}{8}$  inch, milliamperage 25, distance 8 inches, time of exposure 10 minutes. Within 4 days after this dose there appeared an abnormally large number of mitotic figures in the lymphoid tissue of spleen and lymph glands, indicating an acceleration of the proliferative activity of the tissue.

#### EXPLANATION OF PLATES.

##### PLATE 3.

FIG. 1. A splenic nodule, 24 hours after a 20 minute exposure to the small dose of x-rays. Note the abundance of pycnotic cells.

##### PLATE 4.

FIG. 2. A splenic nodule, 24 hours after a 10 minute exposure to the small dose of x-rays. *M*, mitotic figure.

##### PLATE 5.

FIG. 3. A nodule of the mesenteric lymph gland, 4 days after a 10 minute exposure to the small dose of x-rays. *M*, mitotic figure.



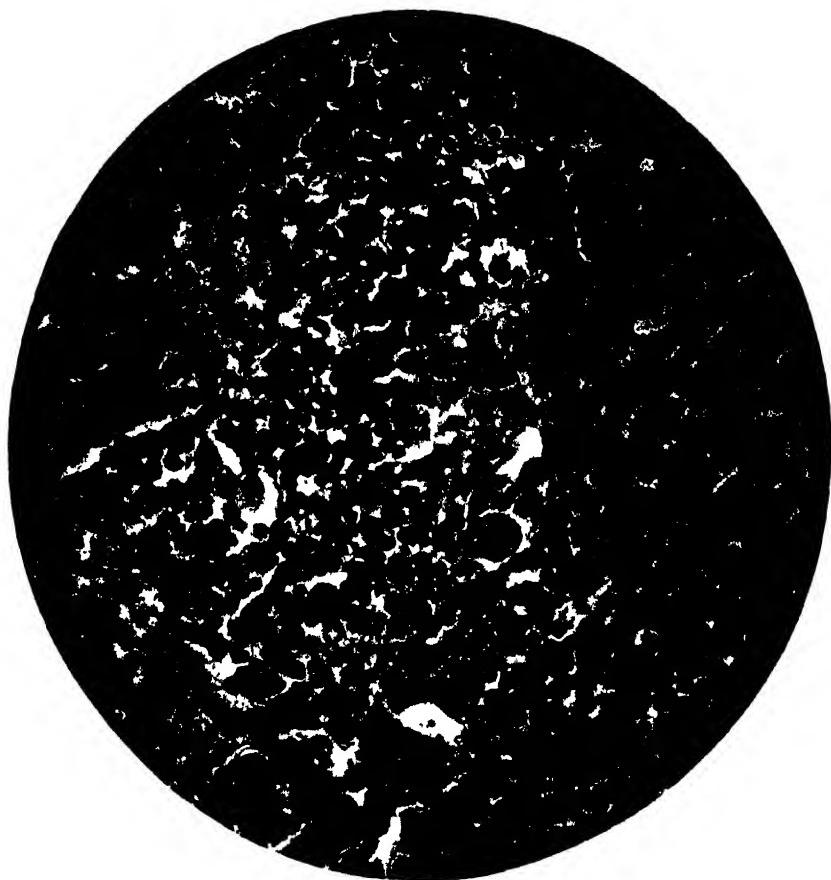


FIG. 1



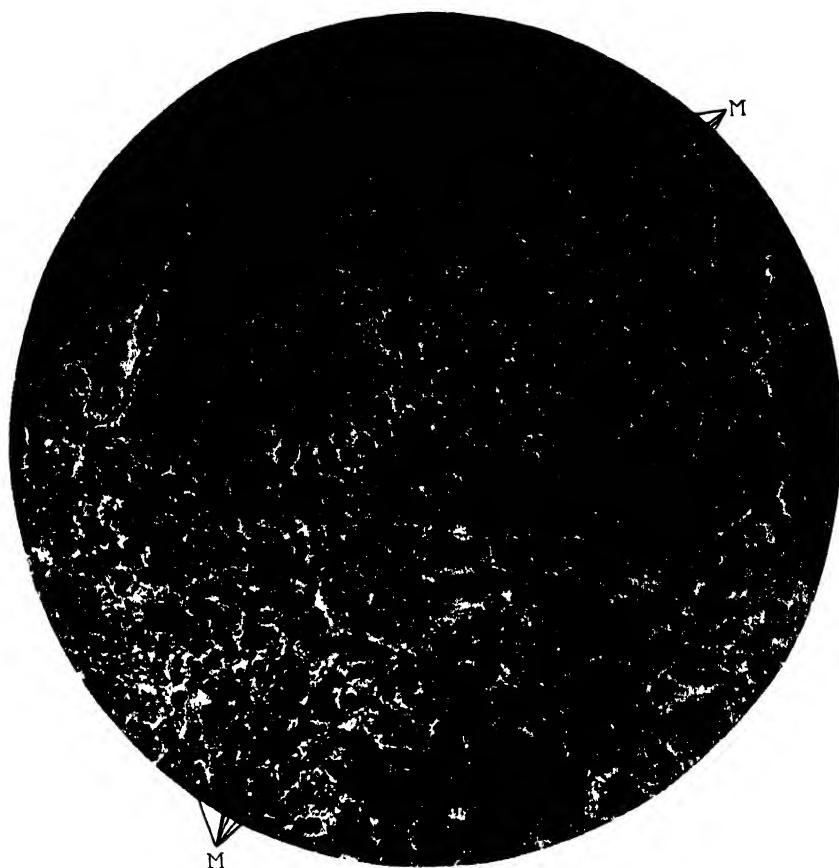


FIG. 2.

(Nakahara and Murphy: Studies on x-ray effects. V.)



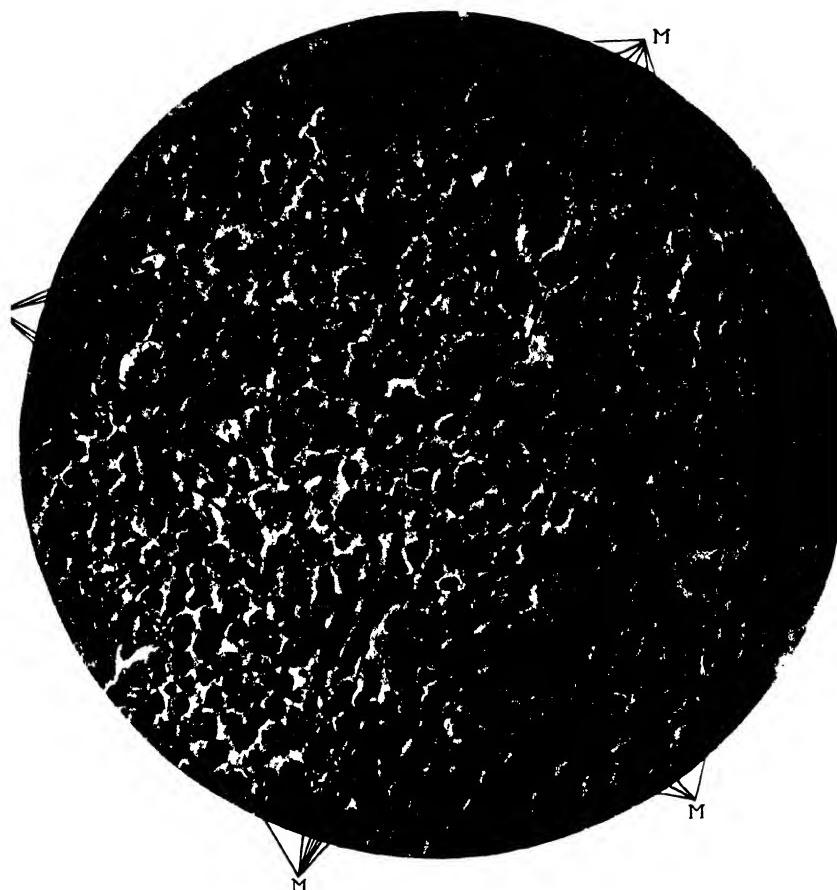


FIG. 3

(Nakahara and Murphy: Studies on x-ray effects. V.)



## TOXINS AND ANTITOXINS OF BACILLUS DYSENTERIAE SHIGA.

By PETER K. OLITSKY, M.D., AND I. J. KLIGLER, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

### PLATES 6 TO 8.

(Received for publication, November 1, 1919.)

The nature of the toxin of the Shiga dysentery bacillus has been studied by a number of bacteriologists, notably by Shiga,<sup>1</sup> Neisser and Shiga,<sup>2</sup> Conradi,<sup>3</sup> Vaillard and Dopter,<sup>4</sup> Rosenthal,<sup>5</sup> Todd,<sup>6</sup> Kraus and Doerr,<sup>7</sup> Flexner and Sweet,<sup>8</sup> Doerr,<sup>9</sup> Pfeiffer,<sup>10</sup> Bessau,<sup>11</sup> and Lüdke.<sup>12</sup> But no agreement as to its precise nature has as yet been reached.

The chief discrepancies in experimental results and in deductions arrived at from them may perhaps be due to difference in method of preparing the toxin. Those who regarded the latter as an endotoxin prepared it by washing off the growth on agar slants with saline solution, shaking, heating to 56° or 60°C., incubating for 24 to 48 hours, and filtering through a Berkefeld candle. Those, on the other hand, who viewed it as an exotoxin obtained it by growing the bacilli in alkaline broth for a period of 2 to 6 weeks and then filtering.

<sup>1</sup> Shiga, K., *Centr. Bakteriol.*, 1te Abt., 1898, xxiii, 599.

<sup>2</sup> Neisser, M., and Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 61.

<sup>3</sup> Conradi, H., *Deutsch. med. Woch.*, 1903, xxix, 26.

<sup>4</sup> Vaillard, L., and Dopter, C., *Ann. Inst. Pasteur*, 1903, xvii, 486.

<sup>5</sup> Rosenthal, L., *Centr. Bakteriol.*, 1te Abt., Ref., 1904, xxxiv, 503; *Deutsch. med. Woch.*, 1904, xxx, 235.

<sup>6</sup> Todd, C., *Brit. Med. J.*, 1903, ii, 1456; *J. Hyg.*, 1904, iv, 480.

<sup>7</sup> Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1905, xviii, 158, 1077.

<sup>8</sup> Flexner, S., and Sweet, J. E., *J. Exp. Med.*, 1906, viii, 514.

<sup>9</sup> Doerr, R., *Das Dysenterietoxin*, Jena, 1907, 30 ff.

<sup>10</sup> Pfeiffer, *Centr. Bakteriol.*, 1te Abt., Ref., *Beilage*, 1908, xlvi, 1.

<sup>11</sup> Bessau, G., *Centr. Bakteriol.*, 1te Abt., Orig., 1910, lvii, 27.

<sup>12</sup> Lüdke, H., *Die Bazillenruhr*, Jena, 1911, 90.

Our studies of the toxic products yielded by the Shiga bacillus have led us to the conclusion that this microorganism growing *in vitro* produces two poisons, one an endotoxin, the other an exotoxin, which can be separated experimentally and can also be shown to attack different anatomical structures of the rabbit and to set up two distinct kinds of pathologic effects.

Shiga first pointed out that the bacillus which bears his name is highly toxic for the rabbit, and this animal has remained the chief one for demonstrating experimentally the pathogenic action of the micro-organism. The Shiga bacillus or its poisonous products induces two kinds of marked lesions in the rabbit; one is localized in the intestine, and the other in the central nervous system.

The first comprehensive study of the lesions in the central nervous system was made by Dopter.<sup>13</sup> He concluded that the central nervous system is usually the seat of serious lesions which may occur in any portion of the system, although the medulla is most often affected. The gray matter, and almost exclusively the anterior horns, show chromatolysis of the neurons in a varying degree and, besides, at times, areas of necrosis which destroy the cellular elements and myelin fibers, leaving scarcely any vestiges of them. At the same time there are an intense hyperemia and even hemorrhages invading the tissue. The white matter is intact. In short, the lesion is that of an acute myelitis, often an anterior poliomyelitis, and sometimes a polioencephalitis as well.

The intestinal lesions were studied by Flexner and Sweet<sup>8</sup> who state that they vary in intensity. The coats of the large intestine are greatly thickened by inflammatory edema, in which case the mucosa is yellowish white and thrown into deep folds and corrugations, or more or less hemorrhage may be associated with the edema. At another time the transverse folds of mucous membrane are affected chiefly; they are swollen, the edges are hemorrhagic, and a pseudomembrane is scattered over the surface. Or, again, the transverse folds are greatly affected and the intervening mucosa is less affected, while patches of swollen and hemorrhagic mucous membrane, covered with a false membrane, appear upon and between the folds. The hemorrhage may extend into the serous coat.

#### *The Exotoxin of Bacillus dysenteriae Shiga.*

*Preparation of the Exotoxin.*—Comparative studies were made of media favoring a high yield of exotoxin as well as the conditions influencing its production. The following protocols are illustrative.

<sup>13</sup> Dopter, C., *Les dysenteries*, Paris, 1909, 75 ff.; *Ann. Inst. Pasteur*, 1905, xix, 353.

*A. Yield of Toxin in Plain Broth and Egg Albumin Broth.*<sup>14</sup>—A quantity of plain meat infusion broth was divided into two lots. To the first was added one-third of its volume of a 10 per cent egg albumin solution. Both lots were adjusted to a pH of 7.8 and were inoculated with the same amount of a 24 hour broth culture of Shiga bacilli, Strain 114 S. After incubating for 5 days the cultures were filtered through a Berkefeld N candle and tested on rabbits weighing 1.500 gm. Table I shows the results.

TABLE I.  
*Yield of Toxin in Plain Broth and Egg Albumin Broth.*

Medium.	Rabbit No.	Amount inoculated intravenously.	Results.
Plain broth.	1	1.0 cc.	Paralysis of posterior extremities in 24 hrs. Died in 36 hrs. No intestinal lesions.
" "	2	0.5	Weakness of anterior extremities in 3 days. Recovered. No intestinal symptoms.
Egg albumin broth.	3	1.0	Paralysis of anterior extremities in 18 hrs. Died in 36 hrs. No intestinal lesions.
" " "	4	0.5	Paralysis of both extremities in 36 hrs. Died in 48 hrs. No intestinal lesions.

*B. Yield of Toxin in Media with Different Degrees of Aeration.*—500 cc. of egg albumin broth were placed in a 2 liter flask giving the medium a surface diameter of 17 cm. and a depth of 2 cm. An equal amount was placed in a 500 cc. flask giving the medium a surface diameter of 7 cm. and a depth of 9 cm. Both lots were inoculated with Shiga bacilli, Strain 109, incubated for 7 days, filtered, and tested (Table II).

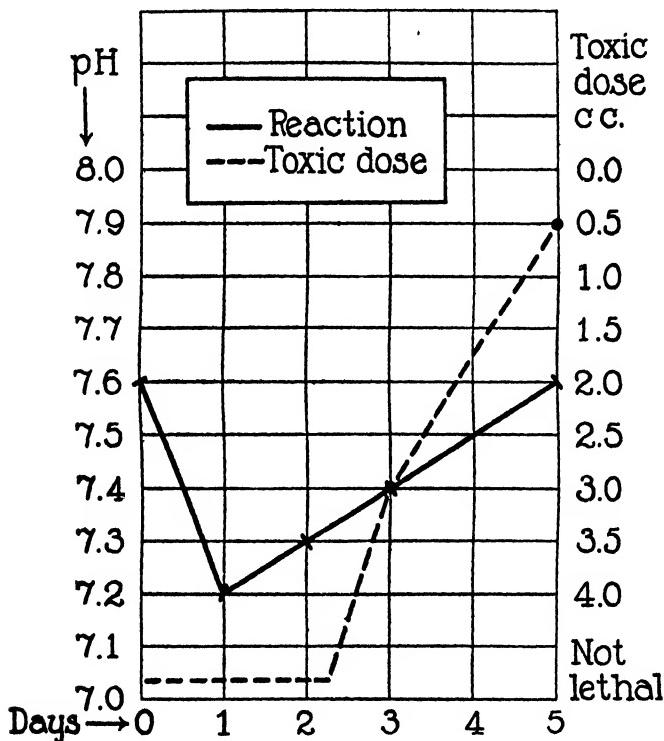
TABLE II.  
*Yield of Toxin in Media with Different Degrees of Aeration.*

Condition of medium.	Rabbit No.	Amount inoculated intravenously.	Results.
Deep.	5	0.05 cc.	No effect.
"	6	0.10	Paralysis in 4 days. " " 2 "
"	7	0.50	
Shallow.	8	0.01	No effect.
"	9	0.05	Paralysis in 1 day. " " 2 days.
"	10	0.10	

<sup>14</sup> It is to be understood where a single protocol only is given that all the experiments were repeated one or more times.

*C. Relation of the Reaction of the Medium to the Yield of Toxin.*—In order to determine the relation of the reaction of the medium to toxin production, small amounts of the culture fluid were tested daily with respect to reaction change and toxic potency.

Lot 15 of egg albumin broth, with an initial reaction of pH 7.6, was inoculated with Shiga bacilli and incubated at 37°C. The results are shown in Table III.



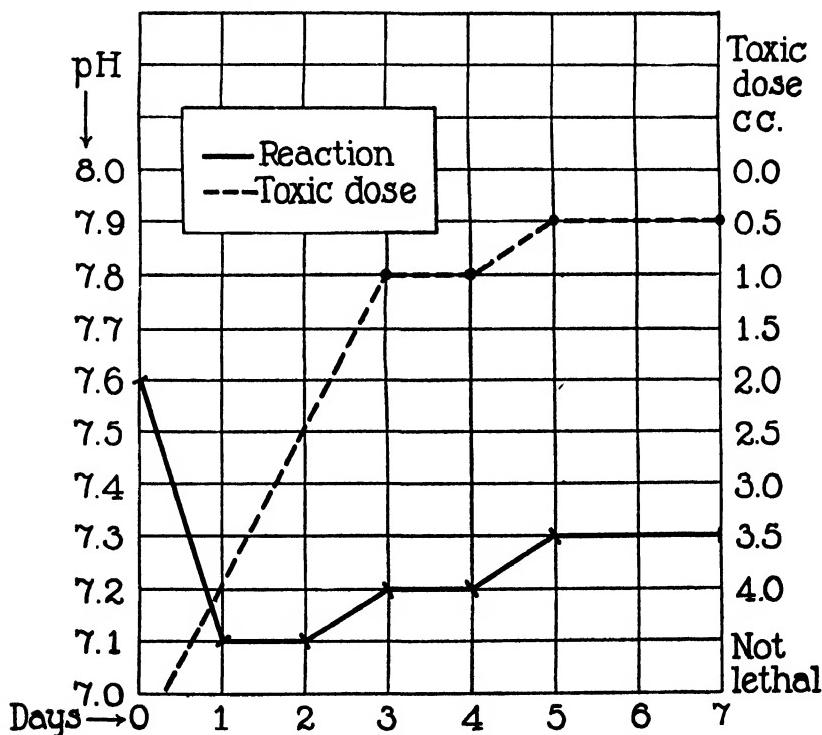
TEXT-FIG. 1. Relation of the pH of the medium to exotoxin production. Broth rendered sugar-free.

It is evident that the changes in the reaction of the medium, the development, in itself, of acid, do not influence the toxicity. But, as is shown in Text-figs. 1 and 2, no toxin is produced in the acid phase; it appears at the beginning of the alkaline phase, and increases thereafter.

TABLE III.

*Relation of the Reaction of the Medium to the Yield of Toxin.*

Length of incubation.	Reaction.	Toxicity.
hrs.	pH	
0	7.6	
24	7.2	None.
48	7.2	"
72	7.3	Minimum lethal dose, 5 cc.
96	7.4	
120	7.4	Minimum lethal dose, 1 cc.
7 days.	7.6	" " " 0.5 "



TEXT-FIG. 2. Relation of the pH of the medium to exotoxin production. Broth not rendered sugar-free.

*D. Relation of the Muscle Sugar Content of the Medium to the Yield of Toxin.*—Lot 14, egg albumin broth, was not rendered sugar-free but otherwise was treated like Lot 15. There was no striking difference in the toxicity, as revealed by comparing Text-figs. 1 and 2. The removal of the muscle sugar does not influence the toxicity.

On the basis of these experiments the procedure adopted for the production of exotoxin was as follows:

Plain meat infusion broth was mixed with one-third its volume of a 10 per cent solution of egg albumin. The latter was prepared by adding one volume of the whites of eggs to nine volumes of distilled water. The mixture was adjusted to pH = 7.6 to 7.8 and quantities of 500 cc. were distributed into 2 liter flasks to permit sufficient aeration and autoclaved for 45 minutes at a pressure of 15 pounds.

This medium was inoculated with one-half an agar slant of a 24 hour culture of the Shiga bacillus and incubated at 37°C. During the period of incubation the contents of the flasks were thoroughly shaken from time to time in order to increase aeration. At the end of 5 days the culture fluid was filtered through a Berkefeld N candle. The filtrate, if proved free from bacteria, constituted the exotoxin.

#### *Nature of the Exotoxin.*

*Pathologic Effects.*—The rabbit is very susceptible and reacts regularly to the action of the exotoxin, the effect depending on the amount injected.

A sublethal dose injected intravenously results in the development of paresis or paralysis of the extremities within 2 to 4 days. Both anterior and posterior extremities may be affected; the former are more frequently involved. The paralytic or paretic stage may endure for 1 to 3 days and may be followed by complete or partial recovery. During this period the animal is apathetic, has no appetite, and loses weight, but intestinal symptoms are either wholly absent or inconspicuous.

A lethal dose injected intravenously results in early paralysis and prostration; that is, within 24 to 48 hours. There is considerable loss of weight. Involuntary evacuations occur but without blood or mucus. Death follows in 1 to 2 days. The autopsy findings are illustrated in the following protocol.

*Rabbit 11 (Figs. 1 to 3).*—Oct. 29, 1917, 2 p.m. Injected intravenously with 0.05 cc. of Exotoxin 2. Oct. 31. Paralysis of posterior extremities. Temperature subnormal. Nov. 1. Prostrated. Incontinence of urine. Stools are frequent, formed, without blood or mucus. 3 p.m. Died.

*Autopsy.*—There were no evident lesions in the intestines (Fig. 3) or other viscera. The cerebrospinal system, however, showed severe lesions. The meninges were free from inflammatory reaction. The gray matter, especially of the medulla and cervical cord, and only slightly of the lumbar cord, was the site of the effects, which consisted of hemorrhage, quite extensive and visible to the naked eye, and, even oftener, multiple, discrete, interstitial hemorrhages as shown in Fig. 1. Areas of necrosis were scattered throughout the gray matter. A perivascular lesion was noted. The neurons showed atrophy or even complete dissolution in limited areas. In places there was chromatolysis, granular degeneration, or caryorrhexis of the cells. The perivascular lesion consisted in an infiltration of small round cells about the arterioles and capillaries, either as a single layer about or in the sheath of the vessels, or less frequently, as a dense, heaped up infiltration, as shown in Fig. 2. The same figure shows an excess of round cells throughout the gray matter. There was a moderate edema of the gray and white matter; otherwise the white matter and nerve fibers were not affected.

The lesions in the nerve tissue are characteristic and constant and agree with those described by Dopter<sup>13</sup> of the nerve injury caused by the whole dysentery toxin, except that he did not describe the perivascular lesion.

The following series of experiments was undertaken to determine whether this toxin is of the nature of an exotoxin.

*Period of Incubation.*—To satisfy the requirements of the class of true toxins, a poison should show a definite period of incubation before the distinctive pathologic effects develop.

*Experiment 1.*—Oct. 8, 1917. Rabbit A injected intravenously with 0.05 cc. of Toxin 1. No effect. Rabbit B injected similarly with 0.1 cc. Oct. 9. Muscular weakness in both anterior extremities. Loss of 115 gm. in weight. Oct. 10. Recovered. Rabbit C injected similarly with 0.5 cc. Oct. 11 (3 days later). Muscular weakness of right anterior extremity. Oct. 12. Flaccid paralysis of this limb. Loss of 175 gm. in weight. Oct. 15. Paralysis improving. Oct. 17. Recovered. Rabbit D injected similarly with 1 cc. Beginning paralysis in 18 hours; complete paralysis of all limbs in 36 hours. Died in 60 hours. None of these rabbits showed intestinal involvement.

The incubation period therefore depends on the dose. The period for one minimum lethal dose is usually from 24 to 48 hours, although

we have noted from fifteen similar sets of experiments that it varies actually from a few hours to 4 days.

*Globulin Fractionating.*—Another point of comparison with the true toxins relates to the globulin fractionating of the poison.

*Experiment 2.*—Jan. 2, 1918. Rabbit A was injected intravenously with a globulin precipitate of Toxin 4. The globulin was purified and 0.2 cc. of a suspension in saline solution, equivalent to four minimum lethal doses, was injected. Jan. 3. Paralysis of both anterior extremities. Prostration. Loss of 70 gm. in weight. Jan. 4. Died.

*Autopsy.*—No visceral lesions. Macroscopic hemorrhages in gray matter of medulla.

*Resistance to Heat.*—An important difference between exotoxin and endotoxin is the thermolability of the former and the thermostability of the latter.

*Experiment 3.*—Several sets of rabbits were injected intravenously with four to ten minimum lethal doses of exotoxin which was heated for varying periods of time at temperatures from 60–90°C. As a control, endotoxin, to be described later, was submitted to similar tests. It was determined that the exotoxin was inactivated or destroyed when heated to 75°C. for 1 hour.

*Production of Antitoxin.*—The toxin yields an antitoxin which will be described in detail later.

*The Law of Multiple Proportions.*—The following experiment is selected from a series to show that Shiga exotoxin conforms to this law.

*Experiment 4.*—10 cc. of Toxin 4 equivalent to 100 minimum lethal doses were mixed with varying amounts of antitoxic serum and incubated for  $\frac{1}{2}$  hour at 37°C. A series of rabbits was injected intravenously and it was found that 0.001 cc. neutralized one lethal unit. In other words, the antitoxic serum contained 1,000 antitoxic units.

Three other toxins were tested with the same antitoxic serum and all were neutralized in the same proportion.

*Specificity of Neutralization.*—A series of control experiments to determine the effect of non-specific sera on the exotoxin shows that no neutralization is obtained.

*Experiment 5.*—Three series of tests were made with normal horse, antitetanic, and antimeningococcic serum. Two to four minimum lethal doses of the exotoxin were mixed with 1 to 5 cc. of these sera and incubated for  $\frac{1}{2}$  hour at 37°C. The

mixtures were then injected intravenously in rabbits. In all instances the typical neurotoxic effect of the toxin appeared.

*Identity of Toxins of Different Strains.*—From the standpoint of identification of the toxin as well as of specific therapy, it is desirable to know whether Shiga bacillus strains from different sources yield the same product. Exotoxins were prepared from the following strains: No. 100 from Newport News, Virginia, on artificial medium 1 year; No. 109 from Poughkeepsie, New York, 2 years; No. 114 F from Japan, more than 10 years; No. 114 S from Germany, more than 10

TABLE IV.  
*Rate of Production of Exotoxin.*

Reaction of medium	Period of incubation.	Amount of filtrate inoculated.	Results.	Classification.
pH	days	cc.		
7.6	0	~		
7.1	1			
7.1	2	5.0	No effect.	
7.2	3	1.0	Paralysis in 48 hrs. No intestinal lesions.	Exotoxin.
7.2	4	1.0	" " 48 " " " "	"
7.3	5	0.5	" " 48 " Died in 72 hrs.	"
7.3	7	0.5	" " 48 " " " 72 "	"
7.5	14	0.25	" " 48 " " " 72 " Intestinal lesions.	" + endotoxin.
7.8	21	0.25	No paralysis. Died in 48 hrs. Marked intestinal lesions.	Endotoxin.

years; and No. 114 T, source unknown, but on artificial medium for many years. The toxins yielded by all these strains were neutralized by the antitoxic serum produced with Strain 109.

It is evident then that strains from widely different sources produce similar exotoxins and that the exotoxin production is a constant phenomenon of the Shiga bacillus, modified slightly if at all by prolonged artificial cultivation.

*Rate of Production.*—The period of incubation of the culture has an important bearing on the production and nature of the toxic product. The prolongation of incubation leads, as will be shown later, to formation of endotoxin, which complicates the results. Table IV,

selected from a series of similar experiments, shows that the exotoxin develops relatively early and as incubation proceeds tends to diminish, while the endotoxin production rises.

To summarize, the Shiga bacillus grown in a favorable medium yields, in the first days of the cultivation, at the beginning of the alkaline phase of its growth, and before bacterial disintegration sets in, a toxic product which appears in the bacteria-free filtrate. This toxic product is precipitated with the globulin fraction of the protein, is relatively thermolabile, is capable of inciting antitoxin formation, is constant in properties, independently of the source of the Shiga culture, and produces in rabbits, after a definite incubation period, typical lesions of the central nervous system without at the same time, in an obvious way, injuring the intestines. In view of its peculiar properties we regard it as an exotoxin and a neurotoxin.

#### *The Endotoxin of *Bacillus dysenteriae* Shiga.*

*Preparation of the Endotoxin.*—The production of the endotoxin of Shiga bacilli does not differ essentially from that of other bacteria. The principle underlying all the methods is that of the autolysis or dissolution of the bacterial cell with the resultant liberation of its intracellular components. Most observations with the Shiga bacillus have been made with endotoxins produced in broth cultures by prolonged incubation (beyond 14 days). When a more rapid yield of endotoxin was desired, the following method was used.

Shiga bacilli were grown in Blake bottles for 24 hours. The growth was then washed off in saline solution, 15 cc. to each Blake bottle, incubated for 2 days at 37°C., and filtered through a Berkefeld N candle. We found that with Strain 100, 2.5 cc. of the filtrate prepared in this manner were lethal for rabbits weighing 1,500 to 1,800 gm.

*Separation of Exotoxin from Endotoxin.*—The technical difficulty of preparing pure endotoxin or exotoxin directly from the Shiga bacillus is great. Usually small amounts of one are found with the other. To establish the integrity of each of the two toxins and their independent action on the rabbit, separation of one from the other was necessary. The removal of exotoxin was accomplished by one of the methods given below.

*Experiment 6. Separation by Heat.*—Toxic Filtrate 16 was prepared by growing Shiga bacilli, Strain 100, in egg albumin broth for 22 days, and filtering.

*Rabbit A (Control).*—Injected intravenously with 1 cc. (four minimum lethal doses) of this filtrate. Paralysis of left posterior extremity after 48 hours, associated with a persistent blood-streaked mucous discharge from the intestines. Died after 4 days.

*Autopsy.*—Typical lesions in the medulla and intestines. Effects due to mixture of exotoxin and endotoxin.

*Rabbit B.*—The toxic filtrate was then heated at 80°C. for 1 hour. 1 cc. (four minimum lethal doses) was injected intravenously in Rabbit B. After 24 hours diarrhea but no nervous symptoms. Died after 4 days.

*Autopsy.*—Large intestine showed hemorrhagic and other lesions; cerebrospinal nervous system normal. Exotoxin destroyed by heat.

*Rabbit C.*—The toxin was also heated to 90°C. for 1 hour. Rabbit C was injected intravenously with 1 cc. (four minimum lethal doses). No effect. Exotoxin and endotoxin both destroyed.

TABLE V.

*Neutralization Experiments with Various Combinations.*

Shiga toxin. Class.	Antiserum.	Result.
Exotoxin.	Antixotoxic.	No effect.
" + endotoxin.	"	Intestinal lesions.
" + "	" + antibacterial.	No effect.
"	Antibacterial (containing antixotoxin).	" "

*Experiment 7. Separation by Neutralization.*—Toxic Filtrate 18 was prepared by growing the Shiga bacilli, Strain 100, in two Blake bottles for 24 hours, washing off with a total of 30 cc. of salt solution, incubating at 37°C. for 2 days, and filtering.

*Rabbit A (Control).*—Injected intravenously with 2 cc. (four minimum lethal doses) of Filtrate 18. After 20 hours paralysis and intestinal symptoms. Died in 24 hours.

*Autopsy.*—Nervous and intestinal lesions. Mixture of exotoxin and endotoxin.

*Rabbit B.*—Injected intravenously with 2 cc. (four minimum lethal doses) of Filtrate 18 to which 1 cc. of antixotoxic serum had been added previous to incubation for  $\frac{1}{2}$  hour at 37°C. After 24 hours severe diarrhea and prostration. No nervous symptoms. Died after 3 days.

*Autopsy.*—Intestinal but no nerve tissue lesions. Neutralization of exotoxin by antixotoxic serum.

Other combinations were tested by injection into rabbits as shown in Table V.

*Nature of the Endotoxin.*

*Pathologic Effects.*—Rabbits are as uniformly susceptible to the effects of the endotoxin as they are to those of the exotoxin. If a sublethal dose is injected intravenously the rabbit shows, after 24 to 48 hours, subnormal temperature, loss of weight, and diarrhea. The stools are frequent and mucoid, occasionally blood-tinged. This condition, during which no nervous symptoms are noted, endures for 2 to 3 days, after which the animal returns to normal.

If a larger but still sublethal, or a lethal dose is injected intravenously the animal reacts within 24 hours with subnormal temperature, considerable loss in weight, and prostration. Severe diarrhea arises, the stools being fluid and containing much mucus and more or less blood. The sensory and motor functions appear normal. The state lasts for 1 to 3 days, after which gradual recovery takes place, or death follows.

At autopsy the peritoneum is dull, its blood vessels are injected, and the peritoneal cavity contains a serous fluid. The small intestines are usually unaffected except that the vessels in the serosa may be injected. Occasionally the ileum is involved in the same extensive way as the large intestine. The walls of the latter are greatly thickened, edematous, injected, and show small discrete hemorrhages. A glairy gelatinous material covers the serous coat. On opening the intestines the contents are found to consist of blood-tinged mucus. The villi are hyperemic; the mucosa is swollen and reveals discrete hemorrhages and small ulcerations. In some instances necrotic areas are seen, and in one instance an area 2.5 cm. wide encircling the cecum was gangrenous. Microscopically destruction of the glandular elements, as well as a superficial general necrosis, is noted (Fig. 4). There is a cellular exudation in the submucosa and considerable edema and degeneration of the muscular layers. In the main, these pathologic effects in the intestine agree with the description given by Flexner and Sweet<sup>8</sup> and others of the intestinal lesions produced by the injection of the whole dysentery toxin.

There are no lesions in the cerebrospinal nervous system. Hence this poison can be regarded, in contradistinction to the exotoxin, as an enterotoxin.

*Resistance to Heat.*—A property common to endotoxins is heat stability. We have determined that Shiga endotoxin is destroyed when heated at 85–90°C. for 1 hour.

*Neutralization by Antisera.*—Antixotoxic serum fails to neutralize endotoxin. Endotoxin, however, is neutralized by an antibacterial serum prepared by actively immunizing horses with Shiga bacilli.

To summarize, the Shiga endotoxin is a definite toxin, probably of intracellular origin, conforming to the properties of the endotoxins as a class. It differs physically and biologically from the Shiga exotoxin. Moreover, the two are separable by various procedures.

### *The Antitoxins of *Bacillus dysenteriae* Shiga.*

That the exotoxin is capable of yielding an antixotoxic serum is shown by the following experiment.

*Experiment 8. Horse A.*—Nov. 12, 1917. Injected intravenously with 5 cc. of Toxin 2 (prepared from Strain 109 grown in egg albumin broth 5 days and filtered) mixed with 1 cc. of polyvalent antidysenteric serum, as described below. Nov. 13. Injected similarly with 5 cc. of toxin but only 0.5 cc. of serum. Nov. 14. Same amount of toxin; 0.1 cc. of serum. Thereafter pure exotoxin was injected, the next series being started 7 days later with 1 cc. The intervals of injection, the increase of dosage, etc., followed the method given by Flexner and Amoss.<sup>15</sup> Jan. 26, 1918. Dose increased to 30 cc., or a total of 80 cc. for the 3 day period of immunization. The horse reacted severely to this amount. Following this, single injections were given at weekly intervals, starting with 20 cc. and increasing slowly to 50 cc. Jan. 28. Trial bleeding; no antitoxic content. Nov., 1918. Trial bleeding; serum showed 1,000 antitoxic units per cc. Feb. 10, 1919. Trial bleeding; serum showed 2,000 antitoxic units per cc. (The basis for computation was one minimum lethal dose.)

Fig. 5 illustrates the results, selected from a series, of one of the neutralizing experiments made with this antitoxic serum. As shown by Rabbit B, the exotoxin is removed from a mixture of endotoxin and exotoxin by neutralization with the antixotoxic serum; but as the endotoxin is unaffected the animal succumbed later and showed the intestinal lesions but no changes in the nervous system.

At this point a study was made of the stock polyvalent antidysenteric serum prepared at The Rockefeller Institute. This serum is ob-

<sup>15</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

tained from horses repeatedly injected with live cultures of Shiga and Flexner bacilli according to the method of Flexner and Amoss.<sup>16</sup> The serum tested was obtained from horses under immunization for several years (one horse 2 years, another 5 years). It was found that although the serum was prepared by injecting the cultures, it contained at least 2,000 antitoxic units per cc. as well as antientotoxic and other antibacterial<sup>16</sup> antibodies.

#### SUMMARY.

With the methods which have been described we have separated an exotoxin and an endotoxin from cultures of the Shiga dysenteric bacillus. The study of the nature and effect of the poison of this microorganism is thus simplified. The two toxins are physically and biologically distinct. The exotoxin is relatively heat-labile, arises in the early period of growth, and yields an antitoxic immune serum. The endotoxin, on the other hand, is heat-stable, is formed in the later period of growth, and is not neutralized by the antitoxic serum. The exotoxin exhibits a specific affinity for the central nervous organs in the rabbit, giving rise to a characteristic lesion—mainly, hemorrhages, necroses, and possibly a perivascular infiltration in the gray matter of the upper spinal cord and medulla. The endotoxin exerts a typical action on the intestinal tract, producing edema, hemorrhages, necroses, and ulcerations, especially in the large intestine.

In dysentery in man the intestinal lesions predominate, but in severe epidemics paralysis and neuritis have been observed (Osler<sup>17</sup>).

These facts become especially significant from the standpoint of the serum therapy of bacillary dysentery. A potent antidysenteric serum should contain antibodies against the exotoxin as well as the endotoxin. That such a serum can be produced in horses has been experimentally demonstrated.

<sup>16</sup> Four lethal doses of endotoxin were neutralized by 0.01 cc. of this serum; on the basis of one lethal unit the serum may be said to contain 400 antientotoxic units. The antibacterial antibodies tested were agglutinins. For some strains of Shiga bacilli the titer reached 1:20,000. In no instance was it less than 1:2,000.

<sup>17</sup> Osler, W., *The principles and practice of medicine*, New York, 1912, 8th edition, 128.

## EXPLANATION OF PLATES.

## PLATE 6.

FIG. 1. Upper cervical region of the spinal cord of a rabbit injected intravenously with Shiga exotoxin. The hemorrhagic lesions in the gray matter, the edema, and the degeneration of the neurons are shown.  $\times 85$ .

FIG. 2. Section of the medulla of a rabbit injected intravenously with Shiga exotoxin. The perivascular lesion is shown. Three capillaries, indicated by arrow-heads, are seen in different stages of round cell infiltration.  $\times 320$ .

## PLATE 7.

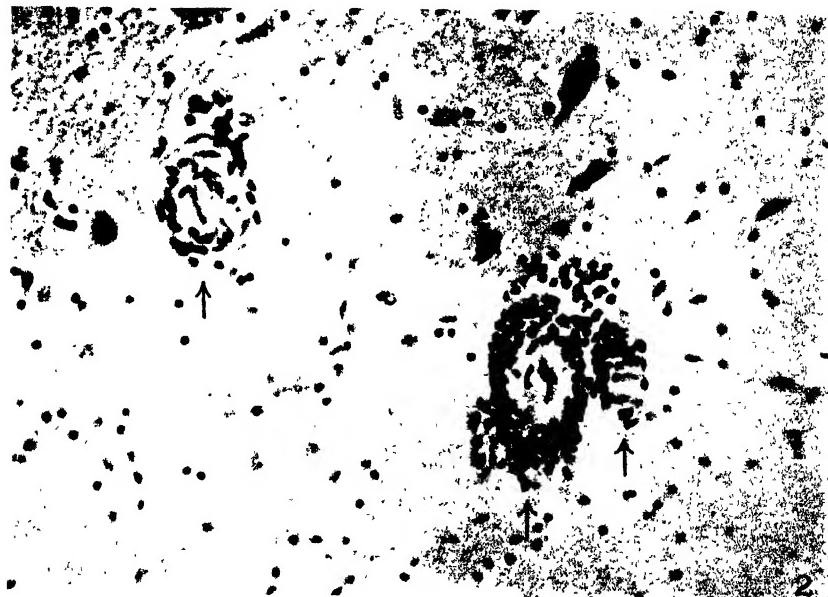
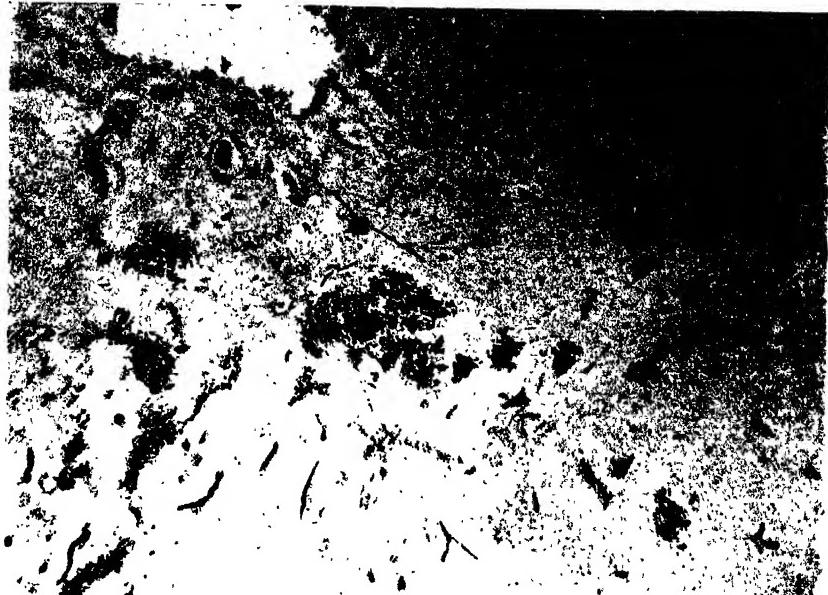
FIG. 3. Intestinal villus of a rabbit injected intravenously with Shiga exotoxin. The villus is not affected.  $\times 72$ .

FIG. 4. Intestinal villus of a rabbit injected intravenously with Shiga endotoxin. The superficial necrosis of the entire villus is evident. Most of the glandular elements are destroyed and the villus is considerably atrophied.  $\times 72$ .

## PLATE 8.

FIG. 5. Rabbits A and B injected intravenously with filtrates containing both exotoxin and endotoxin. Rabbit A, injected with the mixtures of toxins incubated with normal horse serum, shows the effect of unneutralized exotoxin (paralysis and no intestinal symptoms). Rabbit B, injected with the mixtures of toxins incubated with antiexotoxic serum, shows the effect of neutralized exotoxin and unneutralized endotoxin (no paralysis but pronounced intestinal symptoms).





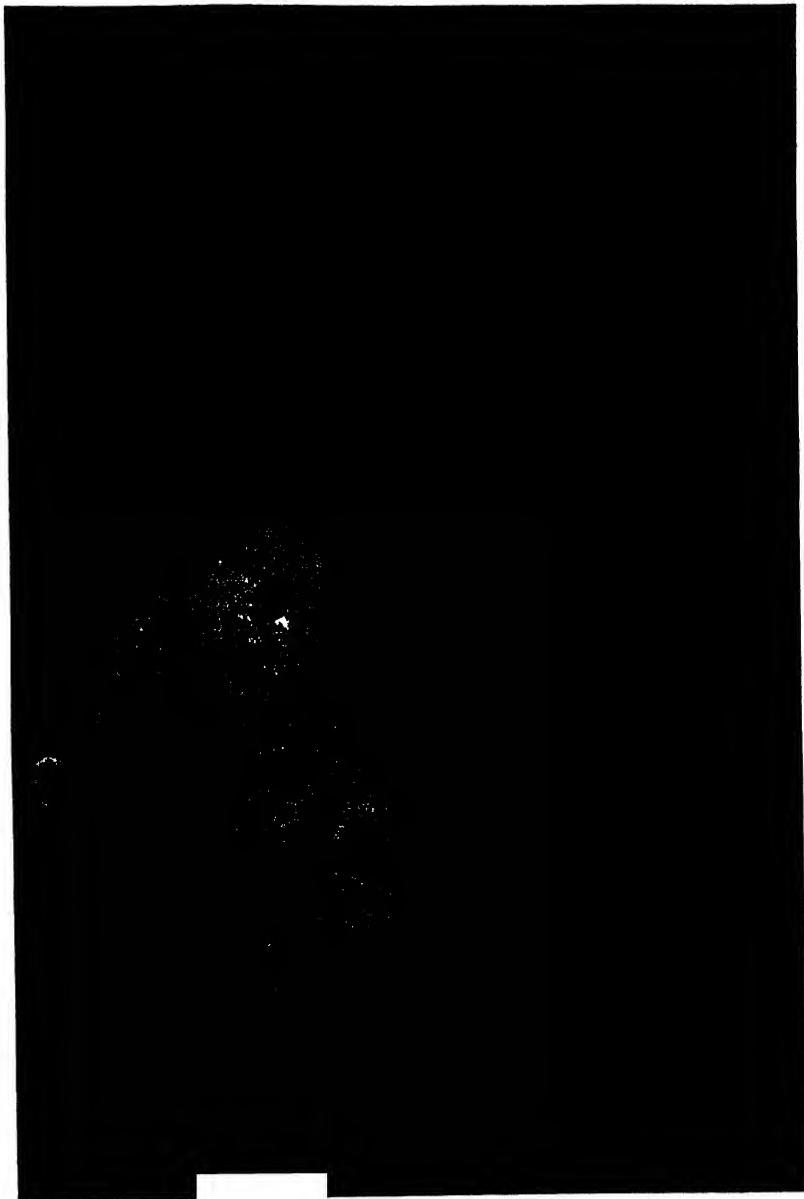
(Olitsky and Kligler: *Bacillus dysenteriae* Shiga.)





(Olitsky and Kligler: *Bacillus dysenteriae* Shiga)





(Oltakay and Kilgler: *Bacillus dysenteriae* Shiga)



## A METHOD OF STANDARDIZING BACTERIAL SUSPENSIONS.

BY FREDERICK L. GATES, M.D.

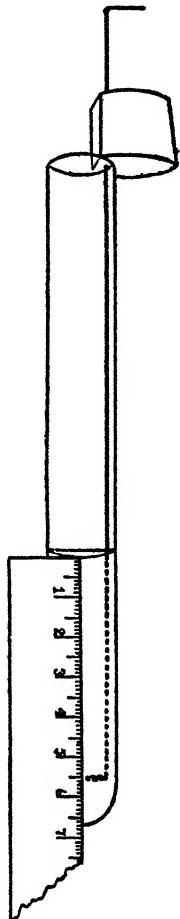
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 23, 1919.)

When bacterial suspensions are used as vaccines or in serum reactions it is necessary or desirable to determine their concentration, at least approximately. Many methods have been advocated. The bacterial count per cc., the weight of the moist or dried bacterial substance, the capacity of a platinum loop, the opacity of various bacterial or chemical suspensions, the volume of the sedimented organisms, all have served as standards in various methods of determining concentration already described. The more accurate methods involve much time and labor; the simpler ones give only a rough approximation of bacterial content.

If a wire loop is gradually pushed down into a suspension of organisms in a test-tube (Text-fig. 1) and viewed by looking down into the tube through its mouth, the depth at which the loop disappears will be determined by the opacity of the supervening column of suspension. If two suspensions of the same organism are compared in this manner, a longer column of the thinner suspension will be required to effect the disappearance of the loop. The lengths of the columns may be measured and compared, and the measurements might be interpreted in terms of bacterial concentration if a suitable standard were determined. Tests with such an instrument show that with a little practice the length of the column of a bacterial suspension that will just hide the loop (the depth of disappearance) can be measured with considerable accuracy. For example, with suspensions of such an opacity that the loop disappears between 1 and 4 cm. below the surface, the zone of most accurate measurement, the depth of disappearance can be read within 1 mm. repeatedly, an error of less than 10 per cent. Within certain limits, neither the diameter of the test-tube nor the size of the wire loop affects

the readings appreciably. Even in the laborious method of counting the organisms the accepted error is often twice as great. If the relation of the depth of disappearance to the concentration could be



TEXT-FIG. 1. The simplest form of the apparatus used to measure the depth of disappearance of a wire loop in a suspension of bacteria.

determined, this would seem to be a quick and simple method of standardizing bacterial suspensions with a minimum of apparatus and manipulation.

It might be supposed that if a suspension containing 1,000 million bacteria per cc. caused the loop to disappear at 2 cm. below the meniscus and this suspension was diluted so as to contain 500 million bacteria per cc., the loop would disappear at 4 cm., or, in other words, that the opacity of a solution would vary directly with its concentration or inversely with the depth of disappearance of the wire loop. This is found not to be the case. In the second instance the loop will disappear at some distance less than 4 cm.; for example, at 2.8 or 3.3 or 3.6 cm. This discrepancy is due to the presence in each reading of a constant which must be eliminated by subtraction in order to bring the readings into ratio with the bacterial concentrations.

The constant appears to be a function of the size and opacity of the individual bacteria in the suspension. Two portions of a suspension of starch grains in cold water, one of which has been heated to boiling, give two parallel series of readings upon successive dilutions, but the constant with the heated specimen is larger than that with the unheated one, corresponding to an increase in size and translucency of the starch grains in the heated suspension. Subtraction of its own constant from each series brings the corresponding readings together and thus indicates that the heated and unheated specimens contain the same amount of starch, which is, of course, the case.

While this constant is the same for any series of readings on the same suspension, it varies with each suspension examined. The problem, therefore, is to eliminate the constant and so to bring opacity and concentration into accord. In practice this is easily done.

A series of readings taken on a suspension at successive dilutions and plotted in graphic form with the readings as ordinates and the corresponding volumes as abscissæ will be found to lie approximately in a straight line. In reality it lies along a flat curve that approaches a straight line as the suspension is further diluted. If the original concentration of the suspension is such that the loop is visible at a distance greater than 1 cm., the error introduced by assuming that successive readings fall along a straight line is not appreciable. For purposes of illustration, therefore, the straight line may be employed, as in Table I, A. The line is plotted in Text-fig. 2, A—A.

Table I, A, represents a series of readings on a bacterial suspension taken at dilutions obtained by adding one, two, three, and four volumes of the diluent to the original volume of the suspension. Inspection of the readings shows that they are not in the same ratios to each other as the corresponding volumes are. But if a constant quantity, in this instance 0.5, is subtracted from each reading, the remainders fall into direct ratios with the volumes, as is seen in Table I, B. Similar results are obtained from the graph. If the straight line is projected across the zero abscissa it crosses it at an ordinate distance of 0.5 cm. A line B-B parallel to A-A and passing through the zero point cuts each volume abscissa 0.5 cm. below the corresponding observed reading for that volume. The

TABLE I.

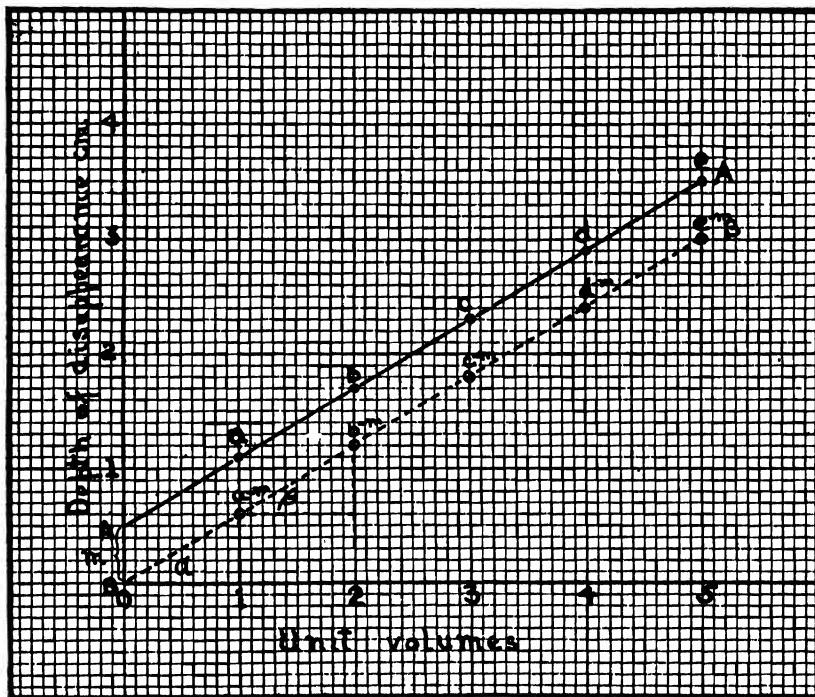
A		B	
Unit volumes.	Depth of disappearance. cm.	Constant.	Corrected readings.
1	1.1		0.6
2	1.7		1.2
3	2.3		= 1.8
4	2.9	0.5	2.4
5	3.5		3.0

corrected reading, divided by its volume, is in each case the tangent of the angle alpha, and the corrected readings are therefore in the same ratios to each other as their corresponding volumes.

It is not necessary to plot the readings or even to find the constant in order to subtract and so to eliminate it, even though it is found to differ with each suspension examined. Each of the observed readings on a single suspension is the sum of the corrected reading and the constant. Let the successive observed readings be  $a, b, c, d$ , etc., and the constant  $m$ . Then the corrected readings are  $a - m, b - m, c - m$ , etc. If any corrected reading is subtracted from a subsequent one the constant is cancelled from the equation,

$$(b - m) - (a - m) = b - a \quad (d - m) - (b - m) = d - b$$

and the remainder is the difference between the observed readings. Obviously the converse is also true and the difference between any two observed readings equals the difference between the corresponding corrected readings. It is convenient to choose such dilutions of the suspension that the difference between successive readings is in



TEXT-FIG. 2. The ordinates represent the depth of disappearance in cm. of a wire loop in a suspension of bacteria. The abscissæ represent the corresponding successive dilutions of the suspension. *A—A*, observed readings, *a*, *b*, *c*, *d*, *e*. *B—B*, corrected readings, from which the constant *m* has been eliminated by subtraction. The corrected readings stand in equal proportion to the corresponding volumes,

$$\frac{a-m}{vol\; a} = \frac{b-m}{vol\; b} = \frac{c-m}{vol\; c} = \frac{d-m}{vol\; d}, \text{ etc.}$$

and may therefore be used to determine the concentration of the bacteria per cc.

each instance equal to the corrected reading for the original concentration of the suspension. This is most easily explained from the graph. Reference to Text-fig. 2 shows that since angle alpha equals angle beta, tan alpha equals tan beta, or

$$\frac{a - m}{\text{vol } a} = \frac{(b - m) - (a - m)}{\text{vol } b - \text{vol } a}$$

Then

$$a - m = \frac{\text{vol } a (b - a)}{\text{vol } b - \text{vol } a}$$

and if

$$\text{vol } b - \text{vol } a = \text{vol } a$$

then

$$a - m = b - a$$

When two observed readings are considered, the difference of whose volumes equals the original volume of the suspension, then the first, subtracted from the second, will give the corrected reading for the original volume of the suspension (Table II).

TABLE II.

Volumes.	Volume <i>a</i> .	Observed readings.	Corrected reading ( <i>a</i> - <i>m</i> ).
2-1	-	1	1.7-1.1 - 0.6
3-2	-	1	2.3-1.7 - 0.6
4-3	-	1	2.9-2.3 - 0.6
5-4	-	1	3.5-2.9 - 0.6

It is thus seen that the corrected reading for any suspension, by which its concentration may be compared with that of a standard suspension of the same organism, may be found by making a reading on the suspension, adding an equal amount of the diluent, making a second reading, and subtracting the first reading from the second. Any error in observation is considerably reduced, however, if the suspension is diluted several times instead of once before the second reading is made, and consideration of the equation

$$a - m = \frac{\text{vol } a (b - a)}{\text{vol } b - \text{vol } a}$$

shows that any dilution of the suspension may be used to obtain the corrected reading  $a - m$ . A concrete example will illustrate the point.

Suppose that in a given suspension whose volume is 2 cc. ( $\text{vol } a = 2$ ) the loop disappears at a depth of 1.2 cm. ( $a = 1.2$ ). The suspension is then diluted by adding, for example, 6 cc. of the diluent, so that the total volume is now 8 cc. ( $\text{vol } b = 8$ ). Let the second reading be 3.6 cm. ( $b = 3.6$ ). Then, substituting in the equation,

$$a - m = \frac{2(3.6 - 1.2)}{8 - 2}, \text{ or } a - m = 0.8$$

The corrected reading for the concentration of the given suspension is 0.8 cm. Now this corrected reading may be directly compared with corrected readings on other suspensions of the same organism. A suspension whose corrected reading is 1.6 cm. contains half as many organisms per cc.; one whose corrected reading is 2.4 cm. contains one-third as many. In this connection it should be remembered that the value obtained for  $a - m$  applies to the original suspension before the dilution required in the test. If the diluted test specimen is to be used for any purpose its corrected reading may be obtained from the equation,

$$b - m = \frac{\text{vol } b(b - a)}{\text{vol } b - \text{vol } a}$$

If several suspensions of the same organism are simply to be compared with one another no further calculations are necessary. In many instances, however, a permanent standard is desirable and it is convenient to translate the opacity into terms of an accepted standard, such as the weight of dried bacterial substance or the number of organisms per cc. The bacterial count is the basis most widely employed, even though it is recognized that similar counts do not always represent equal amounts of bacterial substance, on account of variations in the size of the organisms under varying conditions of cultivation.

Given a corrected reading on the depth of disappearance of a wire loop in a suspension of a microorganism and the corresponding bacterial count, or better, a series of such correlated observations, to reduce the error in counting as well as in estimating opacity, the depth of disappearance of a standard suspension containing, for example, 1,000 million bacteria per cc. is readily found by inverse proportion.

$$\frac{\text{Opacity of standard suspension (unknown)}}{\text{Opacity of given suspension (in cm.)}} = \frac{\text{Count on given suspension (millions per cc.)}}{\text{Count on standard suspension (1,000 millions)}}$$

The opacity (depth of disappearance) of a standard suspension of 1,000 million bacteria of any species having been thus determined, the number of organisms per cc. in any suspension of such bacteria may be readily calculated from the same equation. A complete example follows.

Suppose that the readings quoted on page 111 were made on a suspension of normal type meningococci. It has been found that 1,000 million normal meningococci correspond to a depth of disappearance of 4.2 cm. In 2 cc. of the given suspension the loop disappears at 1.2 cm. When this suspension is diluted to 8 cc. the loop disappears at 3.6 cm. The corrected reading for this suspension is then

$$\frac{2(3.6 - 1.2)}{8 - 2}, \text{ or } 0.8 \text{ cm.}$$

which, substituted in the equation above, gives

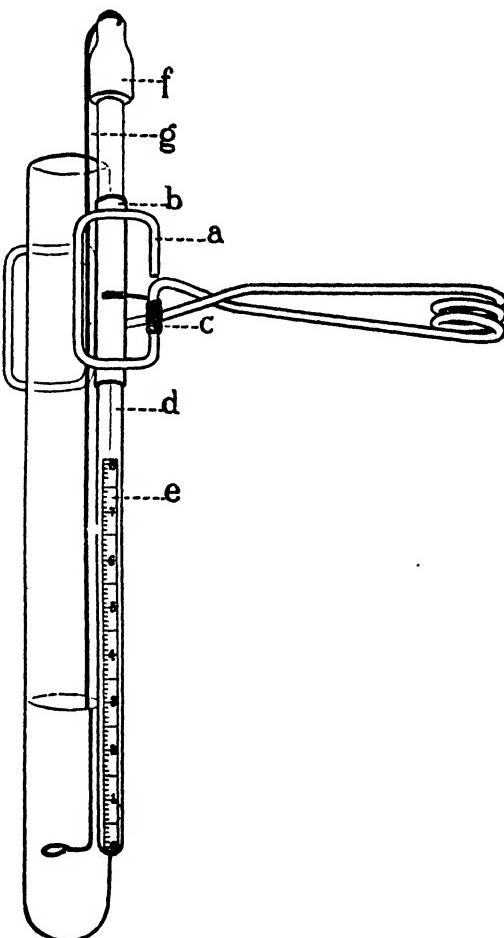
$$\frac{4.2}{0.8} = \frac{x}{1,000}$$

The suspension under examination contains 5,250 million meningococci per cc.

Several simple forms of apparatus have been used to measure the depth of disappearance. The first observations were made with a loop of No. 18 nichrome wire thrust through a cork, as in Text-fig. 1, and measured with a centimeter scale laid against the test-tube. A more convenient instrument, which has proved sufficiently accurate for ordinary use, is shown in Text-fig. 3.

To a wire test-tube clamp (*a*) is soldered a 4 cm. piece of 7 mm. (inside diameter) brass or copper tubing (*b*) slotted in the middle to receive the free end of a small coiled spring (*c*) which presses against an 18 cm. length of glass tubing (*d*), holding the tubing in place, but allowing it to be raised or lowered freely in the tube. A narrow paper centimeter scale (*e*), reading upward, is sealed within the glass tubing, which is surmounted by a stub of heavy walled rubber tube (*f*) to hold the end of the No. 18 gauge nichrome wire loop (*g*). Iron wire may be used, but it rusts and flakes off when repeatedly wet and heated. Nichrome or chromel wire retains its black color and is unaffected by repeated use. The free end of the wire is bent at right angles into a small circle, so that it lies horizontally in the center of the test-tube opposite the zero point on the centimeter scale when the instrument is held in the upright position.

In use a measured quantity of the specimen to be estimated is placed in a sterile test-tube, 1.6 by 16 cm., in the clamp. The wire loop, viewed by looking down into the mouth of the test-tube, is



TEXT-FIG. 3. A convenient modification of the instrument for measuring the opacity of bacterial suspensions.

lowered into the suspension and adjusted until it is just beyond the limit of vision through the fluid; *i.e.*, the opacity of the supervening column of suspension is just sufficient to hide the loop. This

end-point is more accurately observed than one with the loop faintly visible. The depth of disappearance is then read on the centimeter scale at the bottom of the meniscus, care being taken that the test-tube is held perpendicularly, with the meniscus at the level of the eye. A measured amount of the diluent is then added and mixed by agitation, and the second reading is made. The original volume of the suspension (*vol a*), the amount of diluent added (*vol b - vol a*), and the two observed readings (*a* and *b*) give the necessary data for obtaining the corrected reading (*a - m*) on the suspension. This corrected reading, by comparison with the standard for the given organism, figured by inverse proportion as already demonstrated, gives the concentration of the suspension in millions of organisms per cc. A separate sterile test-tube should be used for each suspension examined. The nichrome wire loop is dried and sterilized in a flame. The rubber cap (*f*) permits it to be held out at a right angle for this purpose.

The readings and the calculations, on a slide rule, can be made in 2 or 3 minutes when the standard for the given organism is known. Owing to differences in acuity of vision, a certain personal equation is involved in the reading of the end-point, and the standards should be worked out for each observer by comparison of corrected depth of disappearance readings and the corresponding bacterial count. Once the standards are established, suspensions of the same organism can be estimated rapidly. The method should be found useful in vaccine and serological laboratories in which many suspensions have to be standardized.

#### SUMMARY.

The opacity of a bacterial suspension is measured by the length of the column of the suspension required to cause the disappearance of a wire loop. By a simple formula the measured opacity is translated into terms of the concentration of bacteria per cc., and so made comparable with that of other suspensions of the same organism. An instrument for measuring the opacity of bacterial suspensions is described in detail.

## EXPERIMENTS ON THE NASAL ROUTE OF INFECTION IN POLIOMYELITIS.

By SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, December 3, 1919.)

In this paper we shall describe experiments bearing on the intranasal route of infection in poliomyelitis. That the inciting microorganism or virus of poliomyelitis enters the central nervous system by way of the nasal passages is now generally believed. Moreover, it has been shown that this virus may be present on the nasal mucosa without inducing any signs of disease.

Both healthy and so called chronic carriers of the virus of poliomyelitis occur in man. Wide diversity of view prevails as to the frequency with which carriage of the virus arises and as to the period of persistence of the virus in the carriers. According to one group of observers (Wickman,<sup>1</sup> and Kling, Pettersson, and Wernstedt<sup>2</sup>), healthy and chronic carriers arise numerously during epidemics of poliomyelitis and actually exceed, possibly even many fold, the number of actual cases of the disease. Moreover, the virus may be very persistent in carriers who have recovered from an attack of the disease and be detectable by animal inoculation several months after all the acute symptoms have subsided (Kling, Pettersson, and Wernstedt). However, it should be remarked here that the virus is supposed to undergo gradual deterioration and thus fail in producing typical experimental poliomyelitis, although it is still capable of exciting atypical symptoms and lesions.

Another group of experimenters has come to quite opposite conclusions. Thus Flexner and Amoss<sup>3</sup> who employed excised tonsillar and adenoid tissue for inoculation did not find either the great frequency of occurrence or the long survival of the virus in convalescents implied in the preceding statements. On the contrary, while they found the tonsillar and other tissues infective for monkeys during the early period of the disease in man, they observed no effects, as a rule, from the inoculation of the tissues taken after the acute symptoms had subsided.

---

<sup>1</sup> Wickman, I., Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

<sup>2</sup> Kling, C., Pettersson, A., and Wernstedt, W., *Communications Inst. med. Etat Stockholm*, 1912, iii, 5.

<sup>3</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1919, xxix, 379.

The point of difference involved is important, not only on the general basis of public health considerations, but also because of its bearing on our conceptions concerning the epidemiology of poliomyelitis. The morbidity of poliomyelitis even during severe epidemics is low. This circumstance has been accounted for by assuming either that a relative insusceptibility to the disease exists among general populations, which is no explanation at all, or that because of the wide dissemination of the virus during an epidemic and even in interepidemic periods, an unperceived active immunization of the community takes place. Insusceptibility then is due to specific protection or immunity.

In all instances in man and monkey in which an experimental inquiry has been made, it has been found that when active immunity exists, the blood carries neutralizing or destructive bodies for the poliomyelic virus. No systematic study of the blood of exposed persons who have remained free from obvious poliomyelitis has been made. We conducted a number of tests of the blood of nurses, doctors, and others who had been repeatedly exposed during the severe epidemic in New York State in 1916, without, however, obtaining any clear and decisive results.

The kind of assumed protection just indicated would be general and specific. But experiments of Amoss and Taylor<sup>4</sup> have shown that another kind of potential protective mechanism is demonstrable in man. This device is local and depends upon the presence in the nasal membrane and its secretions of a substance, not yet defined, which possesses the power of neutralizing or otherwise destroying the virus of poliomyelitis. It is suggested that this local process may play an important part in determining the morbidity of epidemic poliomyelitis.

The experiments to be described in this paper relate to several aspects of the problem of intranasal infection in poliomyelitis and bear, therefore, on the preceding discussion. The first experiments to be given concern the question of the power of the nasal mucosa of the monkey to suppress the virus of poliomyelitis directly applied to it; or, in other words, the ascertaining of the period of survival of the virus on the nasal membrane.

<sup>4</sup> Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, **xxv**, 507.

*Fate of the Virus Applied to the Nasal Mucosa.*

An effective means of inducing infection in *Macacus* monkeys is to apply an active poliomyelitis virus to the nasal membrane on a cotton pledge. By virus in this connection is meant the comminuted spinal cord and medulla of a monkey which has suffered from acute experimental poliomyelitis.

Not all monkeys so treated acquire infection; indeed, the percentage of successful inoculations by the nasal route is smaller than by the brain or intracerebral route. The first question to present itself, therefore, is the fate of the virus in animals which may not succumb to intranasal inoculation.

It has already been shown that given an active virus, it can be detected in the nasal membrane by means of subinoculation. That is, if the nasal membrane carrying the virus is excised, ground with sterile sand, suspended in isotonic saline solution, and filtered through a Berkefeld candle, the filtrate will set up poliomyelitis in another monkey into which it is injected intracerebrally and intraperitoneally. The experiments to follow show that with a constant sample of the virus and a uniform mode of inoculation, the survival of the virus on the nasal membrane is irregular and individual.

The virus was one which has long been kept active by monkey passages, and the mode of application was by cotton pledge, which was allowed to remain in a naris for 2 or more hours. Upon removal, the animals were kept under close observation for varying lengths of time, their condition was noted, and the excised nasal membrane, after etherization of the selected animals, employed for obtaining the filtrate, as described above, for purposes of inoculating other monkeys.

*Experiment 1.*—May 25. *Macacus rhesus* A. Cotton pledge carrying the virus remained in a naris over night. 60 hours after the removal of the plug, the animal was killed with ether and the nasal mucosa excised. The filtrate prepared from this membrane was inoculated into *Macacus rhesus* B.

May 28. *Macacus rhesus* B. Received 2 cc. of the filtrate by intracerebral and 5 cc. by intraperitoneal injection. This animal remained well until June 3, when it showed excitement and an ataxic, uncertain gait. The symptoms extended rapidly, paralysis occurred, and the animal died on June 6. The lesions present in the spinal cord and medulla were typical of poliomyelitis.

This experiment shows, therefore, that it is possible to detect the virus by the methods employed at least 60 hours after its application to the nasal membrane. But other tests carried out simultaneously or subsequently on other monkeys killed at the expiration of 40 and 60 hours and 8 days after the removal of the pledge resulted negatively. Hence this experiment may be taken to indicate that the nasal mucous membrane of the *Macacus rhesus* possesses in some instances striking power of destroying or eliminating the virus of poliomyelitis energetically applied to it.

The property of the nasal mucosa to render ineffective, under certain circumstances, an otherwise efficient dose of the virus may be shown in still another way.

The manner of invasion of the central nervous system by the virus of poliomyelitis is still an open question. In view of the difficulties surrounding experimental infection by way of the blood, and the relative ease with which it is accomplished by way of the brain, nasal membrane, sciatic nerve, peritoneum, eye, and even subcutaneous tissue, Flexner suggested that in all instances the passage of the virus from the periphery to the center is ultimately by way of the nerves. According to this view, the virus applied to the nasal mucosa extends along the short olfactory nerve fibers to the brain and spinal cord. A certain amount of support for this mode of infection is supplied by the experiments of Landsteiner and Levaditi<sup>5</sup> and of Flexner and Clark,<sup>6</sup> in which after an intranasal inoculation the brain and cord of the monkeys were removed before any symptoms appeared and injected separately into other monkeys. Flexner and Clark noted that 48 hours after an intranasal inoculation the olfactory lobes but not the medulla and spinal cord might be infectious.

But illuminating as this experimental result is, it must be regarded as the exception rather than the rule. It happens also and perhaps much more frequently that after an intranasal inoculation the virus cannot be detected either in the mucous membrane or in any portion of the central nervous system. The following protocol illustrates this point.

<sup>5</sup> Landsteiner, K., and Levaditi, C., *Ann. Inst. Pasteur*, 1910, xxiv, 833.

<sup>6</sup> Flexner, S., and Clark, P. F., *Proc. Soc. Exp. Biol. and Med.*, 1912-13, x, 1.

*Experiment 2.—Macacus rhesus.* June 1. The cotton pledge carrying the active virus was permitted to remain in the naris for 24 hours. No symptoms had developed by June 5, when the animal was etherized, 88 hours after the tampon had been removed. The right middle turbinate, at the site of the tampon, showed a small hemorrhage into the mucous membrane.

The nasal mucosa and heavy suspensions of the olfactory lobes, postrolandic convolutions, medulla, and cervical and lumbar spinal cord were inoculated separately into the brains of other *rhesus* monkeys. In no instance was infection secured. The control *Macacus rhesus* in which the pledge remained for the same period became paralyzed on the 7th and died on the 9th day. The lesions of the central nervous organs were typical of poliomyelitis.

The virus used in this experiment was active and the procedure adequate. The difference in the results may be attributed to the power of the nasal mucosa in the one and not in the other animal to destroy the virus. This is the more probable explanation, although it is, of course, possible that at the expiration of the 88 hour period the increase of the virus was insufficient to flood the central nervous system so as to be detectable by the inoculation test.

The first view given is, however, supported by another experiment in which the cotton plug carrying the active virus was permitted to remain in the naris only 2 hours. One of the *Macacus rhesus* monkeys developed symptoms, became paralyzed, and the nervous organs showed typical lesions of poliomyelitis. The other showed no symptoms and was etherized on the 16th day. The nasal mucosa, olfactory lobes, and medulla were injected intracerebrally into three *rhesus* monkeys of which none developed symptoms.

#### *Effects of Antiseptics.*

The innate destructive property possessed by the nasal membrane for the virus of poliomyelitis may be regarded as a valuable defensive mechanism. The question has often been raised whether, during an epidemic of poliomyelitis, the application of antiseptics to the nasal mucosa is to be recommended. In the case of chronic meningococcus carriers, the suppression of that microorganism by the introduction of antiseptics directly into the nasopharynx has not been notably successful; and the meningococcus is apparently a much more fragile organism than the microbe of poliomyelitis.

There is a further important consideration. Now that it has been shown that the nasal membranes are themselves defensive, account needs to be taken of the action of antiseptic drugs upon the chemical substances in the membranes upon which their protective function depends. It is fortunate that in the case of poliomyelitis the effects of chemical antiseptics on the virus of poliomyelitis implanted on the nasal mucosa can be directly tested experimentally.

We already possess fair data of the effects of disinfectants on the virus *in vitro*. The effective chemicals chiefly studied are hydrogen peroxide (Flexner and Lewis), formaldehyde (Römer), thymol, potassium permanganate (Landsteiner and Levaditi), and still others. In the experiments to be given the only antiseptics employed were chloramine-T and the oily solution of dichloramine-T, as devised by Dakin and Dunham.<sup>7, 8</sup>

Two protocols only of this series of experiments will be given. In a few instances in which the virus was applied to the nasal mucous membranes, monkeys treated with the dichloramine-T did not become ill or paralyzed, but as in these cases the control animals also failed to come down, it was considered probable that the particular sample of the virus employed for inoculation was ineffective, or that all the animals used were refractory.

*Experiment 3.—Macacus rhesus.* Apr. 16, 5 p.m. Inserted tampon with virus into left naris. Apr. 17, 10 a.m. Removed tampon which was slightly blood-stained. Both nares washed with 1:1,000 chloramine-T solution in water, after which the dichloramine-T in oil was sprayed into the nostrils; twenty-five successive expulsions of the oily solution by hand pressure were made for each side. The spraying was repeated at 12 m. and 2, 4, and 6 p.m. Apr. 18. Spray every 2 hours from 8 a.m. until 6 p.m. Apr. 23. No symptoms had appeared until this date on which the animal showed excitement and paralysis of the left arm. Apr. 25. Animal generally paralyzed and prostrate. Apr. 27. Animal dying; etherized. The lesions in the central nervous organs were typical of poliomyelitis.

<sup>7</sup> Dakin, H. D., and Dunham, E. K., A handbook on antiseptics, New York, 1917, 33.

<sup>8</sup> We are indebted to Dr. E. K. Dunham for the preparation used in our experiments and for advice as to the procedure to follow.

The conditions of the above experiment are severe. The tampon was left in the naris for 17 hours, and injury, as indicated by the blood staining, had been inflicted on the mucous membrane. In the next experiment the conditions are less severe, but the result was not essentially different.

*Experiment 4.—Macacus rhesus.* May 25. Oily dichloramine-T solution sprayed into nares at 2, 4, and 6 p.m. May 26, 8 a.m. Spray as before. 10 a.m. Inserted cotton plug carrying the virus into the left naris. 12 m. Removed tampon and applied the oily spray. Repeated the spray at 2, 4, and 6 p.m. May 29. Animal protects the left leg. June 3. Ataxic; excited. June 4. Extensive paralysis; prostrate. June 8. Dead. The spinal cord and medulla showed typical lesions of poliomyelitis.

The two experiments given do not, of course, show conclusively that the application of antiseptic fluids to the nasopharynx exercises no restraining influence on the multiplication and pathogenic action of the virus of poliomyelitis present there. The conditions of the experiments may well have been too severe to be readily comparable with those arising in man. But account must also be taken of the fact that monkeys sometimes resist the introduction of the virus by means of tampons without any aid to the defensive powers of the nasal membranes whatsoever. If so active an antiseptic agent as the chloramines may thus be ineffective, it would seem that even less could be expected of the indiscriminate chemical solutions often applied by sprays to the nasopharynx.

#### *Blocking Infection via the Nasal Mucosa.*

The mere plugging of the naris of a *Macacus rhesus* with a tampon carrying the active virus of poliomyelitis may not suffice to set up infection. The outcome is determined not only by the degree of activity of the sample of the virus, but also by the strength of the defensive mechanisms possessed by the particular animal. It has been shown that the nasal mucosa is protective, but it appears also that other and deeper mechanisms play a part in supporting or reinforcing the nasal defenses.

One of the deeper mechanisms is the meningeal-choroidal plexus complex, as pointed out by Flexner and Amoss.<sup>9</sup> The latter ascertained that an otherwise ineffective virus tampon could be rendered effective if the integrity of this complex was disturbed as, for example, by setting up within it a temporary chemical inflammation. Various mild chemical irritants suffice for this purpose, but sterile alien serum is highly satisfactory.<sup>9</sup> But the particular point which the next experiments illustrate is not so much the fact of the promotion of nasal infection by the method indicated as the means employed to block infection by way of the nares.

Flexner and Amoss<sup>9,10</sup> have shown also that the introduction of immune poliomyelitic serum by lumbar puncture into the subarachnoid space in monkeys suffices to prevent infection by way of the meninges, blood, naris, etc. The question which was now investigated was whether blocking of the nasal infection could be secured by means of the immune serum injected into the blood.

The first protocol given is that of an unsuccessful attempt to block infection by way of the nares by means of hexamethyleneamine. This drug does display some power of destroying or of inhibiting the development of the virus of poliomyelitis *in vivo* (Flexner and Clark<sup>11</sup>). But its inferiority to immune serum is great, and the experiment which follows can be viewed in that light and also as another control observation.

*Experiment 5.—Macacus rhesus.* Mar. 6. 2 cc. of sterile normal horse serum injected intraspinally. Mar. 7. 2 hour virus-carrying cotton tampon in naris. Mar. 9. 0.5 gm. of hexamethyleneamine in 10 cc. of water given by stomach tube twice a day. Treatment repeated daily for 6 days. Mar. 15. No symptoms. Mar. 16. Animal excited, somewhat ataxic, and protects the right arm. Mar. 22. Condition remained stationary until this date, when the paralysis involved both arms, back, and right leg. Mar. 28. Dead. The lesions present in the central nervous organs were typical of poliomyelitis.

*Experiment 6. (a) Control.—Macacus rhesus.* Feb. 8, 4 p.m. 2 cc. of normal horse serum injected intraspinally. Feb. 9. 2 hour nasal plug carrying the virus. Feb. 18. Arms paralyzed; back and right leg weak. Feb. 19. Pros-

<sup>9</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.

<sup>10</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

<sup>11</sup> Flexner, S., and Clark, P. F., *J. Am. Med. Assn.*, 1911, lvi, 585.

trate. Feb. 20. Etherized. The medulla and spinal cord showed typical lesions of poliomyelitis.

(b) *Test*.—*Macacus rhesus*. Feb. 8, 4 p.m. 2 cc. of normal horse serum intraspinally. Feb. 9. 2 hour nasal plug carrying the virus. On removal the plug was slightly blood-stained. Feb. 12. 5 cc. of immune monkey serum, obtained by pooling the blood from several animals, injected intravenously. This animal was kept under close observation for 2 months during which time no symptoms arose.

*Experiment 7.* (a) *Control*.—*Macacus rhesus*. May 14. 2 cc. of normal horse serum injected intraspinally. May 15. 2 hour virus-carrying plug in naris. May 16. 2 cc. of normal horse serum intraspinally. May 17. Repeated 2 hour nasal plug. May 18. 2 cc. of normal horse serum intraspinally. May 19. Repeated 2 hour nasal plug. May 23. Left facial and right arm paralysis. May 24. Prostrate. May 26. Etherized. The medulla and spinal cord showed typical lesions of poliomyelitis.

(b) *Test*.—*Macacus rhesus*. May 14. 2 cc. of normal horse serum injected intraspinally. May 15. 2 hour cotton plug carrying the virus in naris; 10 cc. of pooled monkey poliomyelic immune serum intravenously. May 16. 2 cc. of normal horse serum intraspinally. May 17. 2 hour plug with virus in naris; 10 cc. of pooled monkey immune serum intravenously. May 18. 2 cc. of normal horse serum intraspinally. May 19. 2 hour plug with virus in naris; 10 cc. of pooled monkey immune serum intravenously. This animal developed no symptoms whatever during several months observation.

*Experiment 8.* (a) *Control*.—*Macacus rhesus*. June 4. 2 cc. of normal horse serum intraspinally. June 5. 2 hour cotton plug carrying virus in naris. June 6. 2 cc. of normal horse serum intraspinally. June 7. 2 hour virus-containing nasal plug. June 8. 2 cc. of normal horse serum intraspinally. June 9. 2 hour virus-carrying nasal plug. June 13. Animal weak; no definite paralysis. June 14. Dead. The medulla and spinal cord showed lesions of poliomyelitis.

(b) *Tests*.—Two *Macacus rhesus* monkeys. Procedure identical in both. June 4. 2 cc. of normal horse serum intraspinally. June 5. 2 hour virus-carrying plug in naris. June 6. 10 cc. of pooled monkey poliomyelic serum intravenously and 2 cc. of normal horse serum intraspinally. June 7. 2 hour virus-carrying nasal plug. June 8. 10 cc. of pooled immune serum intravenously and 2 cc. of normal horse serum intraspinally. June 9. 2 hour nasal plug with virus. June 10. 10 cc. of pooled immune serum intravenously. No symptoms of poliomyelitis developed during the period of observation which extended over several months.

The results of this series of experiments are clear and definite, and show conclusively that even under highly favorable conditions of susceptibility the infection of monkeys with the virus of poliomyelitis applied to the nasal mucosa can be prevented by passive immuniza-

tion of the body by way of the general blood. By this means, therefore, the effective passage of the virus from the nasal mucosa to the central nervous organs can be blocked.

The precise point at which the blocking takes place is in doubt. Two or three possibilities exist: First, after passage of the virus into the blood itself *en route* to the brain and spinal cord. This possibility is small, inasmuch as all the available evidence is against the virus of poliomyelitis reaching the nervous organs from the general blood (Flexner and Amoss<sup>10</sup>). Next, in the nasal mucosa itself, as the blood carrying the immune serum circulates through. There is no way of readily affirming or excluding this idea. It seems improbable, however, that the virus in the interstices of the tissue and especially in the olfactory nerves themselves would have been brought under sufficient influence of the immune serum to have been prevented from multiplying and inducing infection. Third, in the central nervous system itself. In our opinion, this last is the more probable site. All the conditions of the experiments are favorable to the passage of a certain amount of the immune serum into the subarachnoid space. Under the influence of the chemical irritant, both the choroid plexus and the meningeal vessels become more per-vious to protein substances and hence to the immune bodies (Flexner and Amoss<sup>11</sup>). Once the immune bodies reach the subarachnoid space and mingle with the cerebrospinal fluid, infection with the virus of poliomyelitis injected into the blood or meninges themselves is prevented.

There is little doubt that the quantity of immune serum employed in some of the experiments was excessive. Experiment 6 shows that a single intravenous injection suffices to block the development of the virus. But in the experiments in which repeated nasal tampons were employed and in which several injections of normal horse serum were given, it was deemed advisable to maintain the concentration of the immune serum in the general blood. After all, the answer sought by the experiments was whether under conditions of severe inoculation and a highly favorable degree of susceptibility of the animal tested, blocking of the infection could be secured surely by way of the passively immunized general blood.

<sup>10</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499; 1918, xxviii, 11.

**SUMMARY.**

1. The experiments given in this paper, notwithstanding their seeming diversity, relate to the conditions underlying the states of susceptibility and refractoriness to infection with the virus of poliomyelitis applied to the nasal mucosa.
2. Certain monkeys are highly refractory to inoculation *via* the nares with the virus of poliomyelitis, apparently in virtue of a power possessed by the nasal mucous membrane to destroy or otherwise render ineffective the virus applied to it.
3. This property of the nasal mucosa appears to be distinct from any specific protective substance active upon the virus which may occur in the blood.
4. An effective nasal mucous membrane prevents the passage of the energetically applied virus to the brain and spinal cord.
5. The virus of poliomyelitis energetically applied to the nasal mucosa will survive for an undetermined period of time upon an ineffective, but for a relatively brief period of time upon an effective membrane.
6. The protective power possessed by the nasal mucosa is not in itself adequate to prevent infection with the virus introduced upon it, since slight injury to such independent structures as the meningeal-choroid plexus complex favors the passage of the virus from the nose to the central nervous organs.
7. The normal nasal mucosa is, therefore, an invaluable defense against infection with the virus of poliomyelitis; and the number of healthy and chronic carriers of the virus is probably determined and kept down through the protective activities of this membrane.
8. Antiseptic chemicals applied to the nasal mucosa upon which the virus has been deposited exhibit no great protective action and are of doubtful value. Indeed, it is not impossible that to the extent to which they may affect unfavorably the destructive properties of the nasal mucosa, they may be even objectionable.
9. Infection with the virus of poliomyelitis applied to the nasal mucosa under conditions favorable to the extension to the central nervous organs and multiplication there may be blocked or prevented by the injection of poliomyelic immune serum into the blood.

While the exact manner and site of attack of the immune serum upon the virus is somewhat conjectural, when all the available data are considered it seems probable that the meeting place of the virus and immune serum is in the subarachnoid space.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, February 1, 1920, Vol. xxx,  
No. 2, pp. 135-158.]

## ETIOLOGY OF YELLOW FEVER.

### X. COMPARATIVE IMMUNOLOGICAL STUDIES ON LEPTOSPIRA ICTEROIDES AND LEPTOSPIRA ICTEROHÆMORRHAGIÆ.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 18, 1919.)

In a previous paper<sup>1</sup> it was reported that serum from convalescent yellow fever patients has a more or less marked antagonistic effect upon *Leptospira icteroides* derived from certain cases of yellow fever in Guayaquil, as manifested by a positive Pfeiffer phenomenon in the peritoneal cavity of the guinea pig. In a few instances the serum protected the guinea pig from a fatal infection with the organism. A similar result was obtained with the serum of guinea pigs which had recovered from a non-fatal experimental infection with the leptospira.

In the present paper the question of immunity will be more fully discussed, particularly with regard to agglutination, lysis, complement fixation, Pfeiffer's reaction, etc., with immune sera prepared in rabbits and horses by repeated inoculations of *Leptospira icteroides*. Experiments have also been carried out to determine the relation between this organism and *Leptospira icterohæmorrhagiæ* of infectious jaundice by means of cross-immunity reactions *in vitro* and *in vivo*. In the *in vivo* experiments not only passive, but also active immunity has been taken into consideration. A part of this study has already been published in a paper dealing with the leptospira isolated from wild rats and mice in Guayaquil<sup>2</sup> and will not be repeated here.

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.

<sup>2</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

*Production of Immune Sera.*

Monovalent immune sera for each of four strains of *Leptospira icteroides* were prepared in rabbits by injecting the animals intravenously with 2 to 4 cc. of rich live culture, on rabbit serum medium, several successive times at intervals of 7 to 14 days. The animals showed a definite febrile reaction on the 4th or 5th day after the first injection but no other symptoms. Subsequent inoculations produced no perceptible reaction in most of the animals, although some died suddenly, probably owing to the development of anaphylaxis as the number of inoculations increased.

Polyvalent immune serum was produced in a horse by injecting intravenously (jugular vein) gradually increasing amounts of rich live cultures (horse serum medium) of five strains (Nos. 1, 3, 4, 5, and 6) of *Leptospira icteroides*. 20 cc. of the mixture of cultures were given as the initial injection, and subsequent injections were increased up to 200 cc. This dose was maintained for most of the time during immunization. The first inoculation caused a rise in temperature to 40°C. on the 3rd and 4th days, with considerable swelling along the inoculated side of the neck. The animal lost its appetite during the period but regained its normal condition within 5 days. There was no jaundice at any time. Subsequent inoculations caused no perceptible reaction. During a period of 65 days the horse received 2,495 cc. of mixed live cultures in fifteen injections. The following protocol gives the schedule of immunization.

*Horse 2.—Feb. 6, 1919, 20 cc.; Feb. 11, 40 cc.; Feb. 15, 60 cc.; Feb. 19, 125 cc.; Feb. 24, 150 cc.; Mar. 1, 200 cc.; Mar. 6, 200 cc.; Mar. 11, 200 cc.; Mar. 15, 200 cc.; Mar. 19, 200 cc.; Mar. 24, 200 cc.; Mar. 28, 200 cc.; Apr. 2, 200 cc.; Apr. 7, 200 cc.; Apr. 12, 300 cc. First bleeding on Apr. 19, 1919.*

*Effects of Immune Sera upon Leptospira icteroides and Leptospira icterohæmorrhagæ.*

Monovalent immune sera were prepared in rabbits, as described, and experiments conducted to demonstrate the effects of such sera upon homologous and heterologous strains of *Leptospira icteroides* on the one hand and of *Leptospira icterohæmorrhagæ* on the other.

The experiments were designed to throw more light on the relation that may exist, not only among different strains of these organisms, but also between *Leptospira icteroides* and *Leptospira icterohaemorrhagiae* as distinct varieties. The effects of an immune serum are manifold, but we confined our observations to the phenomena of agglutination, immobilization, and disintegration of the organism when mixed with the immune serum *in vitro* and that of the reaction of Pfeiffer following the simultaneous inoculation of the organism and an immune serum into the peritoneal cavity of guinea pigs. Complement fixation tests were also made.

*Agglutination.*—The technique employed for the agglutination tests was as follows:

Rich live cultures, grown at 26°C. for 2 to 3 weeks on rabbit serum medium (one part of serum plus three parts of 0.9 per cent sodium chloride solution), of each strain were selected. 1 cc. of each of a number of cultures was put into a series of small sterile test-tubes, and 0.2 cc. of the fresh immune serum to be tested was added to each tube. Controls with normal rabbit serum accompanied each series of experiments. The culture and immune serum were carefully mixed by gentle shaking and the tubes incubated at 37°C. (water bath) for 2 hours. Examinations of the contents were then made by means of the dark-field microscope.

With pronounced agglutination minute particles could be macroscopically detected. Another examination was made on the tubes after they had been left at room temperature for 96 hours, but the results were identical with those recorded after 3 hours, with the possible exception of a more granular appearance of some of the agglutinated cultures and in extreme instances a thin but definite grayish sediment at the bottom of the test-tubes.

In the present series of experiments there were available three monovalent antisera for *Leptospira icteroides*, comprising Strains 1, 5, and 6, and six for *Leptospira icterohaemorrhagiae*, comprising the Japanese, British, American No. 1, Group 8, Group 11, and Group 30 strains. Against each of these nine monovalent immune sera were tested cultures of five different strains of *Leptospira icteroides*, Nos. 1, 3, 4, 5, and 6, and seven strains of *Leptospira icterohaemorrhagiae*, Japanese, British, French, American No. 1, Group 8, Group 11, and Group 30.

A strong immune serum acting upon the homologous strain of *Leptospira icteroides* agglutinated the organisms quickly into rather large masses, in which they appeared tightly held together. Most of the organisms became immobile, gradually lost their elementary windings, and were soon transformed into stiff, irregularly granular filaments. If the serum was not strong enough to produce these changes, the agglutinated masses were for the most part degenerated, but with several apparently intact immobilized organisms at the periphery. In other instances the agglutinated masses contained a certain number of individuals which were still active, while in still others the organisms in agglutinated masses showed quivering motility in some part of their body. On the whole, agglutination is the first and more constant reaction observed and disintegration the secondary and less constant (Tables I and II).

*Pfeiffer's Phenomenon.*—To complete the observations, Pfeiffer tests were also performed with the immune sera and the various strains of *icteroides* and *icterohæmorrhagiae*. The technique employed was that generally followed. 1 cc. of a given immune serum was mixed in a Petri dish with 1 cc. of a rich live culture and immediately injected into the peritoneal cavity of a normal guinea pig. The peritoneal fluid was withdrawn with a capillary pipette after 30 minutes and 2 hours and examined under the dark-field microscope (Tables I and II).

The reaction of Pfeiffer with the immune sera and homologous strains of *Leptospira icteroides* is prompt and complete. The organisms seem first to be agglutinated into large masses and then to be quickly disintegrated. The phenomenon may be complete within 15 minutes, so that no trace of the organisms can be seen in the peritoneal fluid. The same is true of *Leptospira icterohæmorrhagiae* and the homologous immune sera. A decided increase of actively motile organisms was noticed in the guinea pig peritoneal cavity when a normal rabbit serum instead of a specific immune serum was used.

As shown in Table I, the five different strains of *Leptospira icteroides* reacted to each of the three monovalent immune sera. The intensity of agglutination and disintegration varied somewhat according to whether the strains were homologous or heterologous. Without

TABLE I.

### **Effects of Monovalent Anti-iceroides Sera upon *Leptospira icteroides* and *Leptospira interhemorrhagiae*.**

\* The varying degrees of agglutination and disintegration are recorded by plus signs vertically placed; their absence by a minus sign.  
† indicates positive; ‡ doubtful; — negative.

Normal rabbit serum (control).

TABLE II.



exception the strongest reaction was obtained with the homologous and a less pronounced one with heterologous strains. Disintegration of the organisms was usually complete in the homologous but seldom so in heterologous combinations. Normal rabbit serum exerted neither an agglutinating nor a disintegrating influence upon any of the strains studied. On the contrary, the addition of normal rabbit serum to control tubes kept the organisms active for many days.

That these anti-*icteroides* sera did not agglutinate the various strains of *Leptospira icterohæmorrhagiae* to any marked degree is also shown in this table. There were a few instances in which slight agglutination was observed, but none so marked as that which occurred with the strains of *icteroides*. In contrast to the results obtained with the *icteroides* strains, in no instance was there any disintegration of the *icterohæmorrhagiae* organisms by an anti-*icteroides* serum. The Pfeiffer phenomenon was invariably positive with the anti-*icteroides* serum and the *icteroides* strains, but almost always negative when the anti-*icteroides* serum was tested with the *icterohæmorrhagiae* strains. In two instances there was a suggestive reaction.

Table II presents the results obtained with six different monovalent anti-*icterohæmorrhagiae* sera. The marked difference that exists between the *icterohæmorrhagiae* strains and the *icteroides* strains is shown. Aside from slight variations, the *icterohæmorrhagiae* strains reacted with the *icterohæmorrhagiae* antisera quite generally and strongly and present a marked contrast to the *icteroides* strains, which reacted occasionally and never very strongly. The Pfeiffer phenomenon was positive in all combinations of the anti-*icterohæmorrhagiae* serum with the *icterohæmorrhagiae* group, but only occasionally and slightly with the *icteroides* group. The occurrence of a fairly marked agglutination in certain instances, such as, for example, anti-Japanese serum *versus* No. 1 *icteroides* strain or anti-group No. 11 serum *versus* No. 3 *icteroides* strain, is of considerable interest because of the occurrence of similar weak reactions among the combinations of anti-*icterohæmorrhagiae* sera and certain heterologous *icterohæmorrhagiae* strains. The only reliable differentiation in these instances would seem to be that of the Pfeiffer test. Beyond these few irregularities the behavior of the immune sera towards *icterohæmorrhagiae* and *icteroides* seems to warrant the conclusion that the strains of

*Leptospira icteroides* and *Leptospira icterohæmorrhagiae* form closely related but distinct groups.

*Complement Fixation.*—The technique used in the complement fixation tests was as follows:

Rich cultures of various strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiae* were grown on rabbit serum media, killed by placing the culture tubes in a water bath at 60°C. for 10 minutes, and then used as antigens in the complement fixation tests. Graduated quantities (0.1, 0.05, 0.02, 0.01, 0.005 cc.) of each of the monovalent sera were mixed with a uniform quantity of the antigen (a quantity which had been found to be non-anticomplementary in several doses when tested with 0.1 cc. of complement), and to each tube was added 0.1 cc. of fresh guinea pig serum as complement. The mixtures were brought up to a total volume of 1.5 cc. for each tube by the addition of 0.9 per cent saline solution. After incubation at 37°C. for 1 hour, 0.1 cc. of a 20 per cent suspension of washed sheep corpuscles and 0.1 cc. of anti-sheep amboceptor (rabbit serum), representing three hemolytic units, were added to each tube, the contents thoroughly mixed, and once more incubated at 37°C. for 30 minutes. The results were read after standing for 1 hour. The actual quantity of each antigen used was 0.1 cc. of the killed culture, which exhibited only a slight anticomplementary property when used alone in quantities of from 0.4 to 0.6 cc.

In the majority of instances the reaction was maximum with 0.1 cc. of immune serum, and all tests with 0.005 cc. gave a negative result. The readings of the reaction obtained with 0.1 cc. of the antigens and 0.1 cc. of the immune sera are recorded in Table III.

Complete fixation took place when the immune sera were mixed with the homologous strains, both in the case of *Leptospira icteroides* and in that of *Leptospira icterohæmorrhagiae*. Occasionally, especially among the *icteroides* strains, the fixation was as strongly positive with one or the other of the heterologous strains as with the homologous. There were a number of instances also in which a more or less definite fixation occurred when the anti-*icteroides* sera were mixed with the *icterohæmorrhagiae* strains or the anti-*icterohæmorrhagiae* sera with the *icteroides* strains, some reactions being as strong as +. Generally speaking, however, there was only a limited degree of cross-reaction between *icteroides* and *icterohæmorrhagiae*.

Variations in the fixation reaction were rather marked among the different strains of *icterohæmorrhagiae*, according to the combinations of the immune sera and heterologous strains, some of which showed only a feeble fixation (+) with certain sera.

**TABLE III.**  
*Complement Fixation Tests.*

TABLE III—Concluded.

Serum	Strain of <i>Leptospira icteroides</i>					Strain of <i>Leptospira icterohaemorrhagiae</i>								
	No 1	No 3	No 4.	No 5	No 6	Japanese.	British.	French	American No 1	American No 2	American No 3	Group 8	Group 11	Group 30
Immune serum for <i>Leptospira icterohaemorrhagiae</i> , Group 11 strain.	—	+	—	—	—	+	+	+	+	+	+	+	+	+
Immune serum for <i>Leptospira icterohaemorrhagiae</i> , Group 30 strain.	—	—	—	—	+	+	+	+	+	+	+	+	+	

\* Four plus signs vertically placed indicate no hemolysis, or complete fixation; three plus signs, about 25 per cent hemolysis; two plus signs, about 50 per cent hemolysis; one plus sign, about 75 per cent hemolysis. — indicates almost complete hemolysis, and minus, complete hemolysis, or no fixation.

#### Protective Properties of Immune Sera against *Leptospira icteroides*.

*Monovalent Immune Rabbit Sera.*—Several monovalent immune rabbit sera, some specific for the *icteroides* strains and some for the *icterohaemorrhagiae*, were tested for their protective properties against the experimental infection in guinea pigs with *icteroides* or with *icterohaemorrhagiae*. For this purpose 1 cc. of culture of the strain to be tested was mixed with 1 cc. of immune serum and the mixture injected intraperitoneally into the guinea pig (Table IV).

It was found that Nos. 5 and 6 of the *icteroides* strains were prevented from causing any infection in guinea pigs when inoculated simultaneously, not only with their respective antisera, but also in combination with heterologous antisera. This would indicate that an immune serum prepared with one strain of *Leptospira icteroides* is equally protective against another strain of the same organism. That the protection was specific may be seen from the fact that these two strains of *Leptospira icteroides* caused either a fatal or a rather

TABLE IV.  
Protective Properties of Monoclonal Immune Rabbit Sera against *Leptospira icteroides* and *Leptospira icterohaemorrhagiae*.

Serum.	Strain of <i>Leptospira icteroides</i> .		Strain of <i>Leptospira icterohaemorrhagiae</i> .	
	No. 5.	No. 6.	Japanese.	French.
Normal rabbit serum (control).	One guinea pig died in 7 and the other in 6 days.	Died in 6 days.	Died in 9 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.
Immune Serum 945. Homologous with <i>Leptospira icteroides</i> , Strain 5.	Survived. No symptoms.	Survived. No symptoms.	No	Survived; severe infection with marked lesions.
Immune Serum 942. Homologous with <i>Leptospira icteroides</i> , Strain 6.	Survived. No symptoms.	Survived. No symptoms.	No	Died in 9 days. Typical symptoms and lesions.
Immune Serum 911. Homologous with <i>Leptospira icterohaemorrhagiae</i> , Japanese strain.	Died in 10 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.	Survived. Slight lesions.	Survived. Examination showed a few lung lesions.
			Guayaquil No. 6.	Guayaquil No. 30.
			Two guinea pigs died in 7 days. Typical lesions.	Two guinea pigs died in 8 days. Typical symptoms and lesions.
			Died in 9 days. Typical lesions.	Died in 11 days. Typical symptoms and lesions.
			Died in 8 days. Typical lesions.	Died in 10 days. Typical symptoms and lesions.
			Survived. No symptoms.	Survived. No symptoms.

Immune Serum 947. Homologous with <i>Leptospira ictero-</i> <i>hemorrhagiae</i> , British strain.	Died in 9 days. Typical symp- toms and le- sions.	Died in 12 days. Typical symp- toms and le- sions.	Survived. Slight lesions.	Survived. No symptoms.	Survived; mild infection.
Immune Serum 952. Homologous with <i>Leptospira ictero-</i> <i>hemorrhagiae</i> , American Strain 1.	Died in 11 days. Typical symp- toms and le- sions.	Survived; mild infection.	Survived; mild lesions.	Survived; mild infection.	Survived.
Immune Serum 904. Homologous with <i>Leptospira ictero-</i> <i>hemorrhagiae</i> , Guayaquil Strain 30.	Survived; severe infection.	Died of intercur- rent disease.	Survived. Slight lesions.	Survived. No symptoms.	"

severe infection in guinea pigs when mixed with normal rabbit serum or with one or another of the antisera produced with different strains of *Leptospira icterohæmorrhagiae*. It is noteworthy that the period of incubation when anti-*icterohæmorrhagiae* serum and *Leptospira icteroides* are combined is somewhat prolonged. For example, with Strain 5 of *icteroides* the control animals with normal rabbit serum died in 6 or 7 days, while the guinea pigs receiving the anti-*icterohæmorrhagiae* sera died between 9 and 11 days after the inoculation. This is also true of Strain 6, with which death occurred in 6 days in the control animal and in 10 and 12 days in the animals injected with the anti-*icterohæmorrhagiae* sera. The symptoms and lesions were typical in all instances in which an infection ensued.

The results obtained by reversing the combinations, that is by mixing different strains of *Leptospira icterohæmorrhagiae* with anti-*icteroides* immune sera, show also an unmistakable specificity of the protection afforded by these immune sera. A clear-cut specific protection is shown in the experiments with the Japanese and Guayaquil strains of *icterohæmorrhagiae*; these three strains were effectively neutralized by their homologous immune sera, but not by any anti-*icteroides* immune sera, although the sera seemed to delay death in some instances. The French strain of *icterohæmorrhagiae* was least virulent and did not cause fatal infection in guinea pigs when any one of the immune sera was simultaneously inoculated. The examination of the surviving guinea pigs for the lesions, particularly those in the lungs, after 24 days showed that the guinea pigs which received the anti-*icteroides* sera had numerous old hemorrhagic foci in the lungs, while none or only a few foci were found in those which were inoculated with the anti-*icterohæmorrhagiae* sera.

*Polyvalent Immune Horse Sera*.—A horse was immunized with a mixture of cultures of four strains of *Leptospira icteroides* for a period of 65 days, during which 2,495 cc. of the cultures were injected intravenously as described in the protocol above. At the same time another horse, which had once been immunized for a period of several months in 1918 with various strains of *Leptospira icterohæmorrhagiae*, was injected again, with *icterohæmorrhagiae* cultures, comprising nine strains: Japanese (one strain), American (three strains), French (one strain), British (one strain), and Guayaquil (three strains).

The serum from each horse was then tested for its protective property in guinea pigs against some of the representative strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiae*.

The procedure consisted in injecting into the peritoneal cavity of guinea pigs a mixture of a given quantity of culture (or liver emulsion) of a strain with 1 cc. of immune serum, either full strength or diluted, as indicated in Tables V and VI.

For each dose of the serum two guinea pigs were used in order to determine its titer as closely as possible. Only one guinea pig, however, was used in determining the protective titers of the anti-*icteroides* serum against the *icterohæmorrhagiae* strains or *vice versa*. The amounts of culture (or liver emulsion) used in testing the corresponding antiserum were such as to approximate 500 minimum lethal doses, while for a cross-titration usually about 50 minimum lethal doses, or even 10 minimum lethal doses were chosen. The reason for reducing the number of minimum lethal doses in cross-protection experiments was that in several preliminary tests a larger quantity of culture was found not to be influenced to any great extent by a heterologous antiserum.

The anti-*icteroides* serum protected guinea pigs against approximately 500 minimum lethal doses of *Leptospira icteroides* in doses of 0.001 (for Strain 5) and 0.0001 cc. (for Strain 6). In other words, 1 cc. of this serum neutralizes about 500,000 to 5,000,000 minimum lethal doses of *Leptospira icteroides*, according to the degree of virulence of the culture. On the other hand, at least 1 cc. of the same serum was required to protect guinea pigs against 10 to 50 minimum lethal doses of the Japanese and French strains of *Leptospira icterohæmorrhagiae*. 0.1 cc. of the serum averted a fatal outcome but failed to prevent wholly the infection. The difference in the protective efficacy of the anti-*icteroides* serum against *icteroides* and *icterohæmorrhagiae* is striking.

The anti-*icterohæmorrhagiae* serum exhibited also a marked specific protective property for the *Leptospira icterohæmorrhagiae* strains, neutralizing at least 5,000,000 (for the French) to 500,000 (for the Japanese) minimum lethal doses per 1 cc. Its effect upon the *icteroides* strains was in each case feeble but distinct, since it protected guinea pigs completely against 50 minimum lethal doses per 1 cc.

TABLE V.

*Protective Properties of Polyclonal Anti-icteroides Serum against *Leptospira icteroides* and *Leptospira icterohaemorrhagiae*.*

Anti-icteroides serum. cc.	Strain of <i>Leptospira icteroides</i> .		Strain of <i>Leptospira icterohaemorrhagiae</i> .	
	No. 5. 0.1 cc. of culture (about 500 M. L. D.).	No. 6. 0.1 cc. of culture (about 500 M. L. D.).	Japanese. 0.1 cc. of liver emulsion (about 10 M. L. D.).	French. 0.1 cc. of culture (about 50 M. L. D.).
0.000001	Died in 7 days. Typical symptoms and lesions.	Died in 7 days.	Died in 7 days.	Died in 9 days.
0.000001	Died in 9 days. Typical symptoms and lesions.	" " 10 "		
0.00001	Survived.	" " 9 "	Died in 6 days.	Died in 10 days.
0.00001	Died in 9 days.	Survived.		
0.0001	" " 9 "	"	Survived (!).	Died in 8 days.
0.0001	Survived.	"		
0.001	"	"	Died in 14 days.	Died in 13 days.
0.001	"	"		
0.01	"	"	Died in 10 days.	Died in 13 days.
0.01	"	"		
0.1	"	"	Survived. Had fever.	Survived. Had fever.
0.1	"	"		
1.0	"	"	Survived. Had fever.	Survived. Had fever.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 8 days.

and prevented death, but not infection, when used in a dose of 0.1 cc. It will be seen, therefore, that the respective polyclonal antisera exert a powerful annihilating effect in guinea pigs upon their corresponding type organisms, but there also exists an undeniable, though

feeble, cross-protective reaction, which may be explained by assuming that the two groups of organisms are not altogether alien but are closely related to each other; they may even constitute two subspecies or races.

TABLE VI.

*Protective Properties of Polyvalent Anti-icterohemorrhagiae Serum against Leptospira icterohemorrhagiae and Leptospira icteroides.*

Anti-ictero- hemorrhagiae serum. cc.	Strain of <i>Leptospira icterohemorrhagiae</i> .		Strain of <i>Leptospira icteroides</i> .	
	Japanese. 0.1 cc. of culture (about 500 M. L. D.).	French. 1 cc. of culture (about 500 M. L. D.).	No. 1. 0.1 cc. of culture (about 50 M. L. D.).	No. 5. 0.01 cc. of culture (about 50 M. L. D.).
0.000001	Died in 6 days.	Died in 10 days.	Died in 8 days.	Survived (!).
0.000001	" " 7 "	" " 13 "		
0.00001	" " 6 "	Survived.	Died in 7 days.	Died in 8 days.
0.00001	" " 11 "	Died in 11 days.		
0.0001	Survived.	Survived.	Died in 8 days.	Died in 8 days.
0.0001	Died in 8 days.	"		
0.001	Survived.	"	Died in 7 days.	Died in 9 days.
0.001	"	"		
0.01	"	"	Died in 8 days.	Died in 15 days.
0.01	"	"		
0.1	"	"	Survived; fever.	Survived; mild jaundice.
0.1	"	"		
1.0	"	"	Survived.	Survived.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days.	Died in 7 days.	Died in 6 days.	Died in 7 days.

*Active Immunity.*

Guinea pigs vary considerably in their susceptibility to *Leptospira icteroides*. It sometimes happens that a culture which kills guinea pigs of average susceptibility in a dose of about 0.0001 cc. occasionally

fails to produce a fatal infection in a guinea pig in a dose as large as 0.01 or 0.1 or even 1 cc. Such refractory guinea pigs are rarely met with, but the fact that there exist certain unusually resistant individuals is of great interest. It has also been found that there are exceptionally susceptible individuals which respond to infection with an attenuated culture which no longer attacks the average guinea pig. For example, with certain cultures of *Leptospira icteroides* which had been repeatedly subcultured without passage through the guinea pig for many months, only one out of several animals inoculated with the same culture may come down with typical symptoms. In fact, when the first attempt to restore the virulence of the culture by animal passage failed, a second or third attempt with four or five guinea pigs each time was necessary to obtain a single positive result. These facts furnish possible explanations for certain paradoxical results which are sometimes encountered in determining the state of immunity.

In a discussion of active immunity we may distinguish between that which arises from recovery from a genuine infection and that which follows the inoculation of killed organisms. Animals which, after receiving an inoculation of a sublethal dose of a live culture, do not react definitely, may acquire a state of immunity similar to that of vaccinated animals.

The results with the guinea pigs which had recovered from a more or less pronounced infection after the inoculation of a culture or blood derived from a guinea pig dying of the typical infection with *Leptospira icteroides* will first be described. The tests consisted in the Pfeiffer phenomenon and the effect of the inoculation of different cultures in case of a negative Pfeiffer reaction.

### Series 1.

Four guinea pigs were actively immunized with Strain 5 of *Leptospira icteroides*.

*Guinea Pig Ch 1.*—Nov. 27, 1918. Received 1 cc. of citrate blood from a guinea pig which had been infected with Strain 5 of *Leptospira icteroides* and showed the typical symptoms of *icteroides* infection. After the usual course of infection (fever, slight icterus, albuminuria) the animal became well within 2 weeks. Dec. 19. Received 1 cc. of culture of the same strain. No symptoms followed. Jan. 6, 1919. Received another injection of culture of the same strain, without any perceptible effect.

*Pfeiffer Reaction.*—Jan. 18. A rich culture of the same strain (1 cc.) was injected intraperitoneally. A prompt positive reaction was obtained. Jan. 22. The Pfeiffer reaction was tested with Strain 6, 1 cc. of a rich culture being used. Result positive. On the same day, 4 hours later, another Pfeiffer test was made on this animal with the Japanese strain of *Leptospira icterohaemorrhagiae*, 1 cc. of culture being used. Result negative. This animal died in 23 days with the typical symptoms of *icterohaemorrhagiae* infection.

*Guinea Pig Ch 2.*—The procedure with this animal was similar to that with Guinea Pig Ch 1.

*Pfeiffer Reaction.*—Jan. 18, 1919. Tested with 1 cc. of culture of the same strain. Result positive. Jan. 22. Pfeiffer test with 1 cc. of Strain 6 culture was positive. The same day, 4 hours later, tested with 1 cc. of French strain of *Leptospira icterohaemorrhagiae*. Result negative. This animal died in 14 days with the typical symptoms of *icterohaemorrhagiae* infection.

*Guinea Pig Ch 3.*—The procedure with this animal was similar to that with Guinea Pig Ch 1.

*Pfeiffer Reaction.*—Jan. 18, 1919. Test with 1 cc. of the same strain was positive. Jan. 22. Test with 1 cc. of the British strain of *Leptospira icterohaemorrhagiae* was doubtful. The animal survived.

*Guinea Pig Ch 4.*—The procedure with this animal was similar to that with Guinea Pig Ch 1.

*Pfeiffer Reaction.*—Jan. 18, 1919. With 1 cc. of culture of the same strain test was positive. Jan. 22. With 1 cc. of culture of Strain 6 of *Leptospira icteroides* test was positive. The animal survived.

## Series 2.

Three guinea pigs, out of a large number inoculated with Strain 6 of *Leptospira icteroides*, which suffered more or less severe infection and eventually recovered, were reinoculated with a culture of the same strain once or twice (as described in the protocols below) afterwards, but showed no characteristic symptoms, except a rise of temperature for a day in one (Guinea Pig C 2). Hence they were completely immune to the same strain and were used to test their resistance to certain other strains, including the Japanese strain of *icterohaemorrhagiae*.

*Guinea Pig C 1.*—Dec. 3, 1918. Received 1 cc. of Strain 6 culture of *Leptospira icteroides*. The animal had typical fever and a trace of jaundice, but recovered in about 9 days. Two more injections of 1 cc. of the same culture were given, one on Dec. 19, and another on Jan. 6, 1919.

*Pfeiffer Reaction.*—Jan. 18. With 1 cc. of a rich culture of the same strain, a prompt and positive reaction. Jan. 22. Inoculated with 1 cc. of a rich culture of Strain 5 of *Leptospira icteroides*. Pfeiffer reaction positive. No symptoms within 1 month.

*Guinea Pig C 2.*—Dec. 11, 1918. Received 1 cc. of blood from a guinea pig showing typical symptoms (fever and slight jaundice). The animal eventually

recovered. Jan. 6, 1919. 1 cc. of a rich culture of the same strain was given. There was slight fever for a day, but no infection followed.

*Pfeiffer Reaction.*—Jan. 18. 1 cc. of a culture of the same strain was inoculated intraperitoneally, but no organism could be found in the peritoneal exudate after 30 minutes. Jan. 22. The animal was tested again for the Pfeiffer reaction, with 1 cc. of a rich culture of Strain 1 of *Leptospira icteroides*. There was a complete positive reaction within 30 minutes, and no infection followed the inoculation.

*Guinea Pig C 3.*—This animal was immunized in the same way as Guinea Pig C 2.

*Pfeiffer Reaction.*—Jan. 18, 1919. 1 cc. of a rich culture of the same strain was injected intraperitoneally. Reaction prompt and complete. Jan. 22. 1 cc. of a rich culture of the Guayaquil strain, Group 30, of *Leptospira icterohæmorrhagiae* was inoculated intraperitoneally. When examined after 2 hours the organisms were partially agglutinated, but most of them were actively motile. On the 4th day the temperature rose and remained above normal for 3 days. There was a slight jaundice on the 9th and 10th days, which soon faded. The animal survived. In this instance there existed a mild infection with the *icterohæmorrhagiae* strain.

### Series 3.

Nov. 27, 1918. Three guinea pigs were inoculated with a culture of the Japanese strain of *Leptospira icterohæmorrhagiae*. A second injection was given on Dec. 19, and a third on Jan. 6, 1919. The animals showed a definite but mild infection after the first inoculation, but recovered. No symptoms followed the second or third injection of the same strain.

*Guinea Pig J 1. Pfeiffer Reaction.*—Jan. 18, 1919. With a culture of the Japanese strain, test positive. The animal remained well.

*Guinea Pig J 2. Pfeiffer Reaction.*—Jan. 18, 1919. With a rich culture of Strain 5 of *Leptospira icteroides*, test negative. The animal survived, passing through a moderately severe *icteroides* infection.

*Guinea Pig J 3. Pfeiffer Reaction.*—Jan. 18, 1919. Pfeiffer reaction with a rich culture of Strain 6 of *Leptospira icteroides* not clear-cut. There was a tendency to formation of agglomerated masses, without immobilization or lysis of the organisms. The animal showed mild but typical symptoms of *icteroides* infection after 13 days, but eventually recovered.

### Supplementary Experiment with Anti-*icterohæmorrhagiae* Serum.

Jan. 16, 1919. A number of guinea pigs were inoculated with mixtures of a polyvalent anti-*icterohæmorrhagiae* horse serum prepared by Inada and Ido and different strains of *Leptospira icterohæmorrhagiae* and *Leptospira icteroides*, with a view to determining the protective property of this serum against the *icterohæmorrhagiae* as well as the *icteroides* strains. 1 and 0.1 cc. of the serum were used, mixed with 0.5 cc. of a culture of each strain, and inoculated at once into the peritoneal cavity

of guinea pigs. The protective titer of the serum had previously been tested against the Japanese strain of *Leptospira icterohaemorrhagiae* and the serum had been found to neutralize 1 cc. of the culture in a dose of 0.001 cc. The result of the present experiment showed that the guinea pigs which received the immune serum and cultures of the Japanese, French, American, and Guayaquil strains of *icterohaemorrhagiae* survived, while the control animals without the serum died in 6, 9, 8, and 7 days respectively. There were no symptoms observed in the surviving animals at any time; they were completely protected by the serum in the doses given (1 and 0.1 cc.). All the guinea pigs inoculated with a culture of the British strain of *icterohaemorrhagiae* with or without (one control) the addition of the immune serum survived; that is, the culture employed was apparently avirulent.

The result obtained with the strains of *Leptospira icteroides* was somewhat surprising. The strains used in this group were Nos. 5 and 6, which killed the control animals in 5 and 10 days respectively. Both cultures, however, produced only a temporary febrile reaction when injected together with 1 cc. of the serum and a moderately severe but non-fatal infection with 0.1 cc.

All the guinea pigs surviving in this series of experiments were subjected on Feb. 10, 25 days after the first inoculation, to immunity tests with cultures of various strains. The guinea pig which had received on Jan. 16 the mixture of the Japanese strain and 1 cc. of anti-*icterohaemorrhagiae* serum escaped infection and was inoculated with 0.5 cc. of a culture of Strain 5 of *Leptospira icteroides* on Feb. 10. It died with typical symptoms of *icteroides* infection in 7 days. The guinea pig which escaped infection with the mixture of the French strain and 1 cc. of anti-*icterohaemorrhagiae* serum on Jan. 16 and was injected with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 died in 5 days with typical symptoms of *icteroides* infection. The guinea pig which escaped infection with the mixture of American Strain 1 and 1 cc. of anti-*icterohaemorrhagiae* serum on the first injection and was inoculated with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 finally recovered after a moderately severe *icteroides* infection.

As already mentioned, the guinea pigs which were inoculated on Jan. 16 with 1 and 0.1 cc. of anti-*icterohaemorrhagiae* serum, together with the cultures of Strains 5 and 6 of *Leptospira icteroides*, had a temporary febrile reaction or a moderately severe infection. These animals were injected on Feb. 10, 25 days later, with a culture of the Japanese strain. All except one, which showed only slight lesions in the lungs, died with the typical symptoms of *icterohaemorrhagiae* infection in 8 to 9 days. This would seem to indicate that these animals were protected by the anti-*icterohaemorrhagiae* serum from the fatal outcome of the *icteroides* inoculations, but they were not rendered immune against the Japanese strain of *icterohaemorrhagiae* when tested 25 days afterwards. Also the anti-*icterohaemorrhagiae* serum injected on the first occasion had no perceptible protective action against this strain after a period of 25 days.

## SUMMARY AND CONCLUSIONS.

It has been previously reported<sup>3</sup> that a filterable microorganism belonging to the genus *Leptospira* has been recovered from the blood or organs of human beings suffering from the disease known as yellow fever in Guayaquil, and that the organism, which has been termed *Leptospira icteroides*, induces in certain experimental animals the characteristic symptoms and lesions observed in the patients from whom it was isolated. It has also been previously shown<sup>1</sup> that the serum from patients recovering from an attack of yellow fever in Guayaquil had the power to agglutinate and dissolve the organism when introduced into the peritoneal cavity of a normal guinea pig (Pfeiffer phenomenon). Moreover, the guinea pigs which had once been inoculated with the blood of yellow fever patients without succumbing to the infection, notwithstanding the fact that they had shown a definite febrile reaction after 4 to 5 days, were found to be refractory to a subsequent inoculation of a culture of *Leptospira icteroides*.<sup>4</sup> All these observations pointed to the possible relation of this organism to the disease known as yellow fever in Guayaquil. The demonstration of the filterability of the organism<sup>5</sup> and the transmission of the infection with the same organism by *Stegomyia calopus*<sup>6</sup> have further strengthened the probable etiological significance of the organism in yellow fever.

It was by no means a simple problem to determine the relation existing between *Leptospira icteroides* and *Leptospira icterohæmorrhagiae*. An experiment reported in a previous paper seemed to justify the view that the two leptospires<sup>7</sup> are closely related but not identical, yet it was necessary to exhaust various other modes of differentiation before the distinction between them was firmly established. The present paper continues this phase of the inquiry in further detail.

There have been taken up here the phenomena of agglutination, the reaction of Pfeiffer, complement fixation, the protective properties of various monovalent and polyvalent immune sera, and active

<sup>3</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 87.

<sup>4</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxx, 1.

<sup>5</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

<sup>6</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxx, 401.

<sup>7</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

immunity. As the result of experiments in connection with these immunity phenomena the following data are presented.

Monovalent immune sera prepared by several successive injections in an animal naturally refractory to *Leptospira icteroides* possess the power to agglutinate *in vitro* not only the homologous strains, but also all other strains of *icteroides* tested. On the other hand, a slight effect, or none at all, has been observed when these immune sera have been mixed *in vitro* with various strains of *Leptospira icterohaemorrhagiae*. A similar relation exists between the monovalent anti-*icterohaemorrhagiae* sera and the various strains of *Leptospira icteroides*; that is, there is a slight agglutinating effect in some instances upon the *icteroides* strains, but it is never so strong as that occurring in tests against the *icterohaemorrhagiae* strains. The Pfeiffer reaction gave a sharper differentiation between the two groups, for in most instances the phenomenon was specific for the group. There were occasional doubtful reactions, but not enough to warrant a confusion of the two groups.

Polyvalent immune sera, one specific for *icteroides*, and the other for *icterohaemorrhagiae*, showed a high titer of neutralizing power for the cultures of the homologous groups. It was found, however, that the action of the sera is by no means absolutely specific, because the injection of a sufficient amount of the anti-*icteroides* serum apparently prevented a fatal outcome in a guinea pig inoculated with multiple minimum lethal doses of a culture of *Leptospira icterohaemorrhagiae*, and *vice versa*. The specificity of the serum was demonstrated only when it was used in smaller quantities.

More or less specificity was shown by the complement fixation reaction, but it was not absolute. Weak fixation occurred when the anti-*icteroides* serum was mixed with one or the other of the *icterohaemorrhagiae* strains and *vice versa*, and strong fixation occurred only when the antiserum was mixed with one of the *icteroides* strains. The question naturally arises whether or not this apparent specificity is due to the homology of the serum and not altogether to a difference in genus of the strains. In other words, it is justifiable to question whether all these variations in the degree of intensity of the reaction are not due to strain variations of the same genus. This question is not finally settled by the present investigation, in which only four

*icteroides* and nine *icterohæmorrhagia* strains have been carefully studied. Nevertheless, on the basis of the findings with these thirteen strains, it seems probable that *Leptospira icteroides* and *Leptospira icterohæmorrhagia* are closely allied but are nevertheless distinct in their immunological reactions. Perhaps the difference between the two may amount to that between subspecies or races. It has been pointed out earlier that the pathogenicity of the two is also distinct, inasmuch as *icteroides* produces chiefly icterus and nephritis and *icterohæmorrhagia* hemorrhage and nephritis, the icterus being less and the hemorrhage more prominent in the evolution of the latter infection.

In the study of active immunity—exclusive of vaccination—difficulty has been experienced in the evaluation of the results, owing to the existence of natural resistance to infection among guinea pigs. A guinea pig may recover from the inoculation of *Leptospira icteroides* and then resist a subsequent inoculation with a virulent strain of *Leptospira icterohæmorrhagia*, a condition simulating that brought about by the identity of the two organisms. However, the refractoriness of such an animal to *icterohæmorrhagia* may be due to its natural immunity to it. In the present study, therefore, only those guinea pigs were selected which had reacted typically—though in mild degree—to the *icteroides* infection, in order to determine whether they were subsequently immune to the inoculation of *icterohæmorrhagia*. Indeed, by this mode of experimentation it was found that the guinea pigs which had once passed through an attack of the *icteroides* infection were absolutely immune to a second infection with the same organism but reacted severely and sometimes fatally to a later inoculation of *icterohæmorrhagia*. Although there were a number of instances in which a previous infection with *icteroides* did not confer any perceptible immunity upon the guinea pigs against *icterohæmorrhagia*, another group of guinea pigs showed a considerable resistance to the *icterohæmorrhagia* infection as compared with those which had never been inoculated with *icteroides*. There is not much doubt, therefore, that an *icteroides* attack brings about, in some instances at least, a certain degree of resistance to the *icterohæmorrhagia* infection. Hence the study of the phenomena of active immunity strongly indicates that *icteroides* is closely related immunologically to *icterohæmorrhagia*.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, February 1, 1920, Vol. xxxi,  
No. 2, pp. 159-168.]

## ETIOLOGY OF YELLOW FEVER.

### XI. SERUM TREATMENT OF ANIMALS INFECTED WITH *LEPTOSPIRA ICTEROIDES*.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 18, 1919.)

The high potency attained by a polyvalent immune serum for *Leptospira icteroides* derived from the horse, as revealed in previous experiments on guinea pigs,<sup>1</sup> indicated the possibility that such a serum might be advantageously employed in the treatment of patients suffering from an infection with that organism. In order to ascertain whether or not the serum can exert a beneficial influence upon the course of the infection, several series of experiments were planned in which the guinea pigs were first inoculated with multiple minimal lethal doses of *Leptospira icteroides* culture and then treated with the immune serum at varying intervals afterwards.

Emphasis has been laid upon the fact that guinea pigs vary considerably in their susceptibility to *Leptospira icteroides*,<sup>2</sup> and instances have been cited<sup>1</sup> in which some animals survived after the injection of a large amount of culture, while some succumbed to smaller amounts. Irregularities of this nature were to be anticipated in the present series of experiments, but this source of error was eliminated as much as possible by using the serum in several graduated doses on a corresponding number of animals for a number of days in succession. In some of the earlier series tests were rendered unsatisfactory by the use of a culture which, from unknown causes, failed to kill the control animals, notwithstanding the fact that the same culture had been highly virulent when tested a fortnight previously. In another series the virulence of the culture employed was such that the control

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1920, xxxi, 135.

<sup>2</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.

guinea pigs died within 5 or 6 days after the injection of the culture, and the time for treatment was comparatively brief.

The serum was injected intraperitoneally in amounts of 0.001, 0.01, 0.1, and 1 cc. at intervals of 1, 24, 48, 72, and 96 hours, and even 5, 6, and 7 days, if the animals still lived, after the inoculation of the culture. In the earlier experiments two injections a day of each dose were given (10 a.m. and 4 p.m.), but this practice was soon abandoned in favor of a single daily injection.

In determining the effect of the immune serum upon the infection, the temperature and other characteristic clinical symptoms were noted, and the extent of the lesions was ascertained by killing the surviving animals at a later period, when they were regarded as convalescent. The lesions chiefly considered, although not a wholly reliable index of the severity of the infection, were the hemorrhagic foci in the lungs.

In a series of therapeutic experiments in which the control guinea pigs died within 5 to 6 days, a culture of Strain 5 of *Lepiospira icteroides* being employed, it was found that when injections of the immune serum were begun within 24 hours from the time of the experimental infection (intraperitoneal), no symptoms or lesions developed, provided the animal had received more than 0.001 cc. of the serum. If the treatment was begun 48 hours after infection, it was necessary to inject more than 0.01 cc. in order to prevent their development. At the end of 72 hours, when some of the guinea pigs had begun to show a rise of temperature, more than 0.1 cc. of the serum was necessary to check the progress of the infection, although some animals treated with 0.01 cc. also recovered. After 96 hours, when most of the animals had a high temperature, and some had begun to show a trace of jaundice, the injection of more than 0.01 cc. had a marked influence. The temperature came down to 100-102°F. by the following morning, and no jaundice afterwards appeared in these animals, which recovered within a week. A few guinea pigs, which were slightly icteric at the time of injection of the serum, became more deeply jaundiced on the following day and remained so for a few days, when they began to convalesce. No guinea pig treated within 96 hours with more than 0.01 cc. of serum died.

At the end of 5 days most of the animals which had not been treated with the serum showed jaundice and a decline in temperature, signs of approaching death within 24 hours. A few animals died in the afternoon of that day. To several of these critically ill guinea pigs 0.1 to 1 cc. of the serum was given twice, at 10 a.m. and at 4 p.m., but none of them seemed to be influenced by the injection. Some died on the same day, others on the following day, showing all the typical symptoms. At the end of 6 days several animals which still remained alive without the serum were treated with two doses of 1 cc., but none was saved from death on the same day.

It was found from this series of experiments that the progress of jaundice, after it had existed for 24 hours, could not be checked by the injection of the immune serum.

All the surviving animals were later killed for examination of the lung lesions. The animals treated with a sufficient amount (0.001 cc. or more) within 24 hours showed no lesions, or at most only a few hemorrhagic spots in the lungs. On the other hand, fairly numerous old hemorrhagic foci were found in the lungs of those which had been treated at the end of 48 hour, indicating an infection aborted through the action of the serum. The lung lesions were decidedly more diffuse and numerous in the guinea pigs which received the serum at the end of 72 and 96 hours, although none succumbed to the infection. In the animals which died following treatment with the serum later than 5 days after infection the symptoms and lesions were typical of the infection and indistinguishable from those in the control animals which died without receiving any serum.

In Table I is given another series of experiments carried out with Strain 6 of *Leptospira icteroides*. This culture killed control animals in doses of 0.001, 0.01, and 0.1 cc. within 13, 10, and 7 days respectively, although one guinea pig inoculated with 1 cc. of the culture escaped death after a severe infection. The amount of culture employed throughout the series was 0.5 cc. and was given intraperitoneally. The injections of serum were begun 1 hour after the inoculation and repeated daily for 7 days, in doses of 0.01, 0.1, and 1 cc.

As the table shows, no infection, so far as external manifestations are concerned, took place in any of the guinea pigs injected with the

*Effect of Polyclonal Immune Serum on Experimental Infection with *Leptospira interrogans*.*

June 13, 1919. Thirty-seven guinea pigs were inoculated intraperitoneally, each with 0.5 cc. of Strain 6 of *Leptospira interrogans* culture, representing at least 500 minimal lethal doses. Graduated quantities (0.01, 0.1, and 1 cc.) of Immune Horse Serum 2 were given to the inoculated guinea pigs at intervals of 1, 24, 48, 72, and 96 hours, etc. The object of the experiment was to ascertain the minimal amount of the immune serum which prevents a fatal infection and the maximal time within which the inoculated animals may still be saved by the injection of the serum.

Guinea pig No.	Time elapsed after inoculation. hrs.	Amount of serum injected.	Conditions preceding injection of immune serum.	Subsequent course of infection (after injection of immune serum).		Remarks.*
				No noticeable effects from inoculation.	No symptoms	
x194a	1	0.01	"	"	"	No lesions found.
x194b	1	0.1	"	"	"	" " "
x195a	1	1.0	"	"	"	" " "
x195b	24	0.01	"	"	"	Several old hemorrhages in lungs.
x196a	24	0.1	"	"	"	A few minute foci of old hemorrhages in lungs.
x196b	24	1.0	"	"	"	No lesions found.
x197a	48	0.01	"	"	"	Rather diffuse old hemorrhages in lungs.
x197b	48	0.1	"	"	"	No lesions found.
x198a	48	1.0	"	"	"	A few old hemorrhagic foci in lungs.
x198b	72	0.01	"	"	"	Several old hemorrhagic foci in lungs.
x199a	72	0.1	"	"	"	No lesions found.
x199b	72	1.0	"	"	"	A few old hemorrhagic spots in lungs.

x100a	96	0.01	Temperature 104°F. in a.m. but animal not apparently sick; no jaundice. 1st day of disease (4 day incubation period). Serum given in p.m.	Temperature 105.5°F. after injection of serum. Following a.m. 102.5°; 101.5° in p.m. No further symptoms.	A few old hemorrhagic foci in lungs.
x100b	96	0.1	Temperature 104.5° in a.m.; no other symptoms. Serum given in p.m.	Temperature 104.5° in p.m.; normal next day. Animal well thereafter.	Numerous old hemorrhagic foci in lungs.
x101a	96	1.0	Animal remained well (temperature 102°) after injection of serum.	No change in temperature (102.5°). Animal remained well throughout experiment.	No lesions found.
x101b	120	0.01	Had had high temperature (10°, 103°) 2 preceding afternoons. Temperature not high in a.m. when serum was given (101.5°).	Temperature 103.5° in p.m.; animal, however, seemed well. No further symptoms. Temperature returned to normal following day. Survived.	" " " (natural refractoriness suspected).
x102a	120	0.1	Fever since preceding day (103.5-104°). Temperature in a.m. on serum injection 103°; rose to 104° in p.m.	Temperature next day 103.5° in a.m. and 104.5° in p.m., but on following a.m. 101.5°. Afternoon rise to 104° for 2 more days, then return to normal. Animal survived.	Numerous pale hemorrhagic foci in lungs.
x102b	120	1.0	Temperature 104° in a.m. and 105.5° in p.m. of day before. 104.5° in a.m. of day serum was given in p.m. Jaundice not distinct.	Temperature remained 104.5° in p.m. 102° next a.m. and 101° in p.m. No further febrile or other symptoms. No jaundice developed. Survived.	Pale hemorrhagic foci in lungs.

\* All the guinea pigs surviving were killed on July 4, 1919 (21 days after infection) for examination.

TABLE I—Continued.

Guinea pig No.	Time elapsed after inoculation. days	Amount of serum injected. cc.	Conditions preceding injection of immune serum.	Subsequent course of infection (after injection of immune serum).	Remarks.*
x103a	6	0.01	Fever for 3 preceding days (103.5°, 104°, 104.5°). 102° in a.m. of serum injection. Distinct jaundice; animal very weak, bordering on collapse.	Temperature rose to 104° in p.m. after serum injection. Animal gradually became worse, dying 9 days after infection.	<i>Autopsy</i> .—Extreme jaundice, and general hemorrhage, especially in lungs.
x103b	6	0.1	Fever for 3 preceding days (103°, 104.5°, 104.5°). Temperature 104° in a.m. of injection. Slight trace of jaundice.	Temperature 104.5° in p.m. after injection of serum. Fever disappeared next day. Animal well throughout remainder of experiment. Survived.	Extensive foci of old hemorrhages in lungs.
x104a	6	1.0	Fever for 3 preceding days (104.5°, 106°, 105°). 103° in a.m. of serum injection. Mild jaundice.	Temperature same p.m. 104.5°, but only 101° next day. Jaundice persisted 3 days longer, then faded. Survived.	Extensive foci of old hemorrhages in lungs.
x104b	7	0.01	Fever for 2 preceding days (105°, 102.5°). Temperature 104° on day of injection. Jaundice slight.	Temperature 104° after serum injection, but 102° next 3 days, returning slowly to normal. Jaundice disappeared in few days.	Rather numerous old hemorrhagic lesions in lungs.
x106a	7	0.1	Fever 3 preceding days (106°, 104.5°, 103.5°). Temperature 102.5° in a.m. of serum injection. Distinct jaundice. Serum given in p.m.	Temperature 103.5° same p.m., 102.5° next a.m., 103.5° p.m., 102° next a.m., 101° p.m. Jaundice persisted 3 days, then faded. Survived.	Very marked hemorrhagic lesions in lungs.

x107a	7	1.0	Fever 3 preceding days (105°, 104.5°, 103°). Temperature 102° in a.m. of serum injection. Marked jaundice. Serum given in p.m.	Temperature 103.5° in p.m.; 101-101.5° on days following. Jaundice increased for 2 days after injection of serum, disappearing 5 days later. Survived.	Hemorrhagic foci in lungs less marked than in preceding animal.
x107b	7	1.0	High temperature for 4 afternoons preceding (104°, 104°, 103°, 104°), but less in mornings (102.5°, 104°, 102°, 102°). Temperature 102.5° in a.m. of serum injection. Jaundice intense for 2 days. Serum given in p.m.	Temperature 103.5° same p.m.; 100° next a.m. Jaundice and collapse. Died 8 days after inoculation.	<i>Autopsy</i> .—Typical lesions.

Guinea pig No. (control).	Amount of culture. cc.	Course of infection.		Remarks.*
		Onset.	Course of disease.	
x192a (control).	0.001	Incubation of 9 days, followed by fever for 4 days (104°, 102°, 105.5°, 103°F.). Jaundice noticed on 3rd day of disease; increased in intensity for 3 days following. Death occurred, with temperature 98°, 13 days after inoculation.		<i>Autopsy</i> .—Typical lesions.
x192b (control).	0.01	Incubation of 4 days, followed by period of fever for 5 days. Jaundice developed in 7 days and became extremely intense within 24 hrs., remaining so until death, which occurred on 10th day after inoculation, with temperature of 96.5°.	" " "	" " "
x193a (control).	0.1	Incubation period 4 days, followed by fever for 48 hrs. (104.5°, 103.5°), then collapse, with temperature 99° on 8th day. Jaundice appeared on day before death, which occurred 7 days after inoculation.	" " "	Killed. Extensive hemorrhagic lesions in lungs.
x193b (control).	1.0	Temperature 103.5° on 4th day after inoculation. Fluctuated for 48 hrs. longer, but was at no time higher. Jaundice appeared on 6th day, increased in intensity rapidly during next 48 hrs., then receded, having disappeared 4 days later. Animal survived.	" " "	

serum within a period of 72 hours from the time of inoculation with the culture. All remained well.

At the end of 96 hours most of the animals had a temperature of 103-104.5°F. in the morning. The injections of the serum were made in the afternoon. The temperature remained high after the injection but dropped gradually the following day, and the animals recovered rapidly.

At the end of 5 days most of the animals had had a high fever for 2 days. The serum was given in the afternoon. Two of the three guinea pigs treated began to improve the next day, one had a high temperature for a day longer, but all eventually returned to the normal condition.

At the end of 6 days the animals had begun to show more or less jaundice and had had fever for 3 days. One guinea pig which received 0.01 cc. of the serum died 9 days from the time of infection, while those which received 0.1 and 1 cc. recovered.

Experiments were made at the end of 7 days, when the animals had had fever for about 3 days, and a slight decline had begun in the morning. In some there had been jaundice for 48 hours. The serum was injected in the afternoon. The temperature the following morning was quite high in most animals, and in some it lasted for 2 to 3 days, while in others it went down rapidly. Jaundice increased in intensity in some, but gradually disappeared within several days. All recovered except one, which was near collapse when 1 cc. of serum was injected and which died the next day.

Of the surviving guinea pigs, no lesions were found in the lungs of those treated with the serum within 1 hour; and in each group of three guinea pigs treated 24, 48, 72, or even as late as 96 hours (during the incubation period) there was one animal in which no lesion was present. In others, irrespective of the amount of serum given, there were a certain number of hemorrhagic spots in the lungs, even when treated with the serum within 24 hours. In one instance there was no lung lesion in a guinea pig treated with 0.01 cc. after 5 days. These irregularities are explained by the existence of a considerable variation among individual animals in their susceptibility to *icterooides* infection.

On the whole, the results obtained in this series admit of only one interpretation; namely, that the immune serum, when injected during the period of incubation, prevents further development of the infection, and that when used in the early stage of the disease it is capable of preventing a fatal termination of the infection. On the other hand, when guinea pigs are inoculated with a highly virulent culture, the injection of the serum at a period of the disease when jaundice has existed for some time and the animal is nearing collapse seems to have no benefit. Undoubtedly the virulence of the strain employed for the experiments has considerable bearing upon the efficacy with which the immune serum may be used in the late stage of the disease. The less virulent the culture, the greater are the chances of benefit from the injection of the serum after the clinical symptoms have manifested themselves, as shown by the favorable results obtained in the last series of experiments just recorded. The fact must be emphasized, however, that even with a less virulent strain the serum has no beneficial effect when given to a guinea pig which is in a condition bordering on collapse (fall of temperature, with intense jaundice).

#### SUMMARY.

The use of a polyvalent immune serum of high potency in the treatment of an experimental infection of guinea pigs with *Leptospira icteroides* was found to be of definite advantage in checking the progress of the infection. When administered during the period of incubation the serum was found capable of completely preventing the development of the disease, although on subsequent examination hemorrhagic lesions of greater or less number and extent were found in the lungs of the guinea pigs which survived. Moreover, the serum modified the course of the disease and when used in the early stages of infection prevented a fatal outcome. Employed at a later stage, however, when jaundice and nephritis had been present for several days and the animal was near collapse, the serum had no perceptible beneficial effect. This was, of course, to be expected in view of the incidence of various pathological phases of this disease—nephritis, hepatitis, and other toxic symptoms in succession. In man the clinical manifestations are more gradual and distinct than in the

guinea pig,<sup>3</sup> yet the yellow fever patient whose temperature is subnormal, and who has reached the stage of hemorrhages from the gums, nose, stomach, and intestines, and of uremia and cholemia, would seem to have little or no chance of deriving benefit from the use of a specific immune serum. This latter assumption would probably hold irrespective of the relation which *Leptospira icteroides* proves to have to the etiology of yellow fever.

<sup>3</sup> Noguchi, H., *J. Expt Med.*, 1919, xxix, 547.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, February 1, 1920, Vol. xxxi,  
No. 2, pp. 201-207.]

## CRESCENTIC BODIES IN ÆSTIVO-AUTUMNAL MALARIA; THEIR MIGRATION AND ATTACHMENT TO THE SURFACE OF THE RED CORPUSCLE.\*

By MARY R. LAWSON, M.D.

(*From the Laboratory of Dr. Mary R. Lawson, New London.*)

PLATES 13 AND 14.

(Received for publication, October 1, 1919.)

### *Æstivo-Autumnal Parasites.*

Since 1879, when malarial parasites were first described, the consensus of opinion has been that the parasites are intracellular, and that each parasite, when young, enters a red corpuscle, staying there until it dies or segmentation takes place, the destruction of the corpuscle corresponding with the segmentation of the parasite. But this is not the case. All malarial parasites are extracellular, that is they are attached to the external surface of the infected corpuscle, and each parasite destroys several red corpuscles. One may trace the destruction of corpuscle after corpuscle by the migrating parasites of æstivo-autumnal infections.

The parasites usually present in the blood of æstivo-autumnal infections are the ring-forms and crescentic bodies. When round bodies are present, they are usually developmental forms of the crescent. The crescent may present an ovoid appearance (1) when bent on itself and viewed from the convex side and (2) when contracted. When the parasite is contracted, the width of the body is increased.

### *Crescentic Bodies.*

The shape of the crescent is similar to that of a caterpillar; the extremities are generally blunt, but one or both may be pointed. That the body of the parasite is wider than it is thick is illustrated by the appearance of one when bent on itself (Figs. 6, 37, and 49).

\* Aided by a grant from The Rockefeller Institute for Medical Research.

*Attachment of the Crescent to the Red Corpuscle.*—The parasite is extracellular and wraps itself around the corpuscle as a worm wraps itself around a berry (Figs. 1 to 10 and 36). The attachment of these bodies to the external surface of the red corpuscle is demonstrated by the following facts. (a) When attached to a corpuscle and seen in profile, the poles of the parasite may be observed extending beyond the periphery of the infected corpuscle in many instances (Figs. 1 to 3, 5 to 8, and 10). (b) When viewed from above, one occasionally sees the poles of the attached crescent extending beyond the periphery of the corpuscle (Figs. 9, 12, 26, 30, 32 to 34, and 38 to 40), and in these instances it is often possible to trace the outline of the corpuscle crossing the body of the parasite (Figs. 32, 34, 38, and 39). In rare instances the body proper of the crescent may be seen protruding beyond the periphery of the infected corpuscle (Fig. 48; the dotted line shows where the corpuscle crosses the parasite). (c) Occasionally the corpuscle may be seen situated between the body proper and the clubbed end of a crescent bent on itself (Fig. 37).

Ordinarily the corpuscle appears only on one side of the crescent, especially after the corpuscle has been decolorized; but occasionally one may see the corpuscle projecting on either side of the crescent. In these instances the parasites show but little curving and one views them from above (Figs. 13 to 16, 18, 19, 26 to 28, 31, 33, 35, 39, and 40). In the majority the hemoglobin of the corpuscles is more or less intact. Under such circumstances, where the parasite is attached to its full extent, the picture is what one would expect, as the healthy corpuscle is more elastic than the parasite, and spreads out in all directions when pressure is exerted.

#### *Hemoglobin Mounds.*

The mounds of hemoglobin substance which the parasite encircles with its cytoplasm for the purpose of attachment and assimilation are easily demonstrated when seen at the periphery of the parasite (at o in Figs. 1 to 3, 5, 6, 29, 32, 35, 47, and 49). These mounds are usually decolorized before the corpuscle itself, but occasionally they may be seen not yet decolorized, in connection with parasites attached to corpuscles showing varying stages of dehemoglobinization, and to corpuscular skeletons (Figs. 41, 43, and 46, at o).

*Migration of the Crescentic Bodies.*

Crescentic bodies go through migratory stages similar to those of other malarial parasites, and it is possible to find all the stages in one film, especially if the infection is a heavy one and quinine has not been given.

*Evidence of Migration.*—That the crescentic bodies destroy more than one red corpuscle is strongly suggested by the following facts. (a) Heavily pigmented parasites may be found attached to newly invaded red corpuscles. By newly invaded, I mean instances where the hemoglobin appears to be as yet unaltered by the action of the attached parasites (Figs. 1 to 5, 8 to 10, 12, 14 to 20, 22 to 28, and 56). The pigmentation of these parasites is evidence of previous attachments. (b) In the same film pigmented parasites are observed on red corpuscles showing varying degrees of dehemoglobinization (Figs. 6, 7, 11, 21, 29 to 40, 47 to 49, and 53). (c) Pigmented parasites occur on corpuscular skeletons or remnants of red corpuscles which have been altered by the action of the attached parasites (Figs. 41 to 46). These skeletons may also be seen free from parasites. (d) In the same film pigmented parasites are found free from red corpuscles (Figs. 50 to 52, 54, and 55). The pigmentation of these parasites is evidence of previous attachments.

Occasionally a crescent is seen in the act of attaching itself to a fresh red corpuscle before it has conformed itself to the curved surface of the corpuscle (Fig. 56; a careful examination of this figure will show clearly the attachment of the parasite to the corpuscle).

I have never observed the migratory stages in connection with the crescents after the administration of quinine. Possibly the continued use of quinine may inhibit the migration of the crescents without causing their immediate destruction, as they may be present in the blood for some time after quinine has been used without any appreciable anemia resulting. Migration of the crescentic bodies is less frequently observed in the blood than is migration of the young forms of the æstivo-autumnal parasite. This finding might be explained by the fact that the infection is usually recognized before the appearance of the crescents and vigorous treatment instituted. But the large amount of pigment which most of these bodies contain

would indicate that they may do considerable damage before their destructive action is restrained.

A heavily pigmented, full grown parasite attached to a red corpuscle the hemoglobin of which is intact, or nearly so, should suggest that the parasite must have obtained that pigment from another source, and that source was undoubtedly another red corpuscle. It seems to me that this is convincing evidence of parasitic migration.

There is considerable evidence of the migration of the crescentic bodies in the observations of several writers. Free crescents and heavily pigmented crescents attached to healthy appearing red corpuscles have been described and frequently illustrated. Osler<sup>1</sup> pictures free pigmented crescents. Laveran<sup>2</sup> pictures free pigmented crescents and a pigmented parasite attached to a healthy appearing red corpuscle. Mannaberg<sup>3</sup> pictures free crescents and pigmented crescents attached to healthy appearing red corpuscles. Canalis<sup>4</sup> states that the crescents decolorize the red corpuscles and finally become free in the blood, and he illustrates<sup>5</sup> free crescents and pigmented crescents attached to healthy appearing red corpuscles. Thayer and Hewetson,<sup>6</sup> in describing crescents, state that in some instances all trace of the corpuscle may be absent, and Thayer<sup>7</sup> gives figures showing a pigmented crescent on a corpuscle that has not been decolorized, and two free crescents. Manson<sup>8</sup> pictures a pigmented crescent attached to a healthy appearing red corpuscle. Marchiafava and Bignami<sup>9</sup> show a pigmented cres-

<sup>1</sup> Osler, W., quoted from Laveran, A., *Paludism*, translation by Martin, J. W. London, 1893, 41, Fig. 7, F and H.

<sup>2</sup> Laveran, A., *Paludism*, translation by Martin, J. W., London, 1893, 18, Fig. 3.

<sup>3</sup> Mannaberg, J., *The malarial parasites*. A description based upon observations made by the author and by other observers, translation by Felkin, R. W., London, 1894, Plate 2, Figs. 51 and 52, Plate 4, Figs. 35 to 42, 53, and 54.

<sup>4</sup> Canalis, P., quoted from Thayer, W. S., and Hewetson, J., *The malarial fevers of Baltimore*, *Johns Hopkins Hosp. Rep.*, 1895, v. 24.

<sup>5</sup> Canalis, P., *Studi sulla Infexione malarica. Sulla varietà parassitaria delle forme semilunari di Laveran e sulle fibrill malariche che da esse dipendono*, *Arch. sc. med.*, 1890, xiv, 75, Plate 3 C, Figs. 4, 5, and 12, 3 B, Figs. 7 to 9.

<sup>6</sup> Thayer, W., and Hewetson, J., *The malarial fevers of Baltimore*, *Johns Hopkins Hosp. Rep.*, 1895, v. 93.

<sup>7</sup> Thayer, W. S., *Lectures on the malarial fevers*, New York, 1897, Plate 3, Figs. 29, 33, and 41.

<sup>8</sup> Manson, P., *Tropical diseases: a manual of the diseases of warm climates*, London, Paris, New York, and Melbourne, 2nd edition, 1900, 14, Fig. 9.

<sup>9</sup> Marchiafava, E., and Bignami, A., *Malaria*, in Stedman, T. L., *Twentieth century practice*, New York, 1900, xix, Plate 2, Figs. 54, 61, 64, and 65.

cent attached to an apparently healthy red corpuscle, as well as free crescents. Celli<sup>10</sup> pictures a pigmented crescent attached to a healthy appearing red corpuscle and a free crescent. Brumpt<sup>11</sup> pictures a free crescent and Thompson<sup>12</sup> heavily pigmented crescents attached to red corpuscles whose hemoglobin appears to be intact, as well as crescents free from red corpuscles.

#### EXPLANATION OF PLATES.

##### PLATE 13.

Magnification,  $\times 1,684$ .

Figs. 1 to 5. Heavily pigmented crescents attached to the periphery of healthy appearing red corpuscles, the hemoglobin of which appears to be intact. Hemoglobin mounds encircled by the cytoplasm of the parasites may be seen at o.

Fig. 6. A heavily pigmented crescent attached to a slightly decolorized red corpuscle. Hemoglobin mounds may be seen at o and an attaching filament from the cytoplasm of the parasite may be seen at x. This filament extends beyond the periphery of the infected corpuscle.

Fig. 7. A heavily pigmented crescent attached to a slightly decolorized red corpuscle. The poles of the crescent may be seen extending outside the periphery of the corpuscle.

Fig. 8. A heavily pigmented crescent attached to a healthy appearing red corpuscle, the hemoglobin of which appears to be intact. An attaching filament extending beyond the infected corpuscle is seen at x.

Fig. 9. A heavily pigmented parasite attached to a healthy appearing red corpuscle. The clubbed ends of the crescent may be seen bent over the edge of the infected red corpuscle.

Fig. 10. A heavily pigmented parasite attached to a healthy appearing red corpuscle. The clubbed end at the lower part of the picture is seen extending beyond the periphery of the infected corpuscle.

Figs. 11 and 12. Heavily pigmented parasites attached to fairly healthy appearing red corpuscles.

Fig. 13. Crescentic body attached to a red corpuscle showing Schüffner's granules.

Figs. 14 to 21. Heavily pigmented parasites attached to healthy appearing red corpuscles.

Fig. 22. A heavily pigmented crescent attached to a healthy appearing red corpuscle. A flagellum taking the nuclear stain may be seen at x.

---

<sup>10</sup> Celli, A., Malaria, New York, 1901, 45, Figs. N and R.

<sup>11</sup> Brumpt, E., Paludisme, Précis de parasitologie, Paris, 2nd edition, 1913, Plate 1, Fig. 14.

<sup>12</sup> Thompson, D., The origin and development of gametes (crescents) in malignant tertian malaria: some observations on flagellation, etc., *Ann. Trop. Med. and Parasitol.*, 1914-15, viii, 85, Plate 5, Figs. 12 to 14 and 28 to 32.

FIGS. 23 to 29. Heavily pigmented crescents attached to red corpuscles apparently containing a normal amount of hemoglobin. Fig. 29 shows hemoglobin mounds at o.

FIG. 30. A heavily pigmented crescent attached to a corpuscle partly dehemoglobinized. What is probably an attaching filament, arising from the cytoplasm of the parasite, may be seen extending from x to x.

FIGS. 31 to 35. Heavily pigmented crescents attached to red corpuscles in varying stages of dehemoglobinization. Hemoglobin mounds may be seen at o. In Figs. 32 to 34 the poles of the parasites may be seen to extend beyond the periphery of the corpuscles to which the parasites are attached. In Fig. 33 one of Maurer's rings may be seen in the infected corpuscle at x. In Fig. 35 an attaching filament arising from the cytoplasm of the parasite may be seen at x.

#### PLATE 14.

Magnification,  $\times 1,684$ .

FIG. 36. A heavily pigmented crescent wrapped around a decolorized red corpuscle. The external relation of this parasite to the corpuscle is clearly shown.

FIG. 37. A heavily pigmented crescent attached to a decolorized red corpuscle. At x the corpuscle may be seen between the body of the parasite and one of the poles. This parasite could not be within the infected corpuscle.

FIGS. 38 and 39. Heavily pigmented crescents attached to decolorized red corpuscles. The poles of the attached parasites may be seen extending beyond the periphery of the unbroken outline of the infected corpuscles. The outline of the corpuscles may be traced through the bodies of the attached parasites.

FIG. 40. A heavily pigmented crescent attached to a decolorized red corpuscle. At A the crescent is bent over the periphery of the infected corpuscle.

FIGS. 41 to 46. Heavily pigmented, healthy appearing crescents attached to corpuscular skeletons. Note the general semilunar appearance of the skeletons. Hemoglobin mounds may be seen at o. Fig. 42 shows the crescent bent on itself. Attaching filaments arising from the cytoplasm of the crescent and attached to the corpuscular skeleton may be seen at x in Fig. 46.

FIG. 47. Pigmented crescents attached to decolorized red corpuscles. Hemoglobin mounds may be seen at o.

FIG. 48. A pigmented crescent attached to a decolorized red corpuscle. The body of the attached crescent extends beyond the periphery of the decolorized corpuscle. The dotted line marks where the corpuscle crosses the parasite.

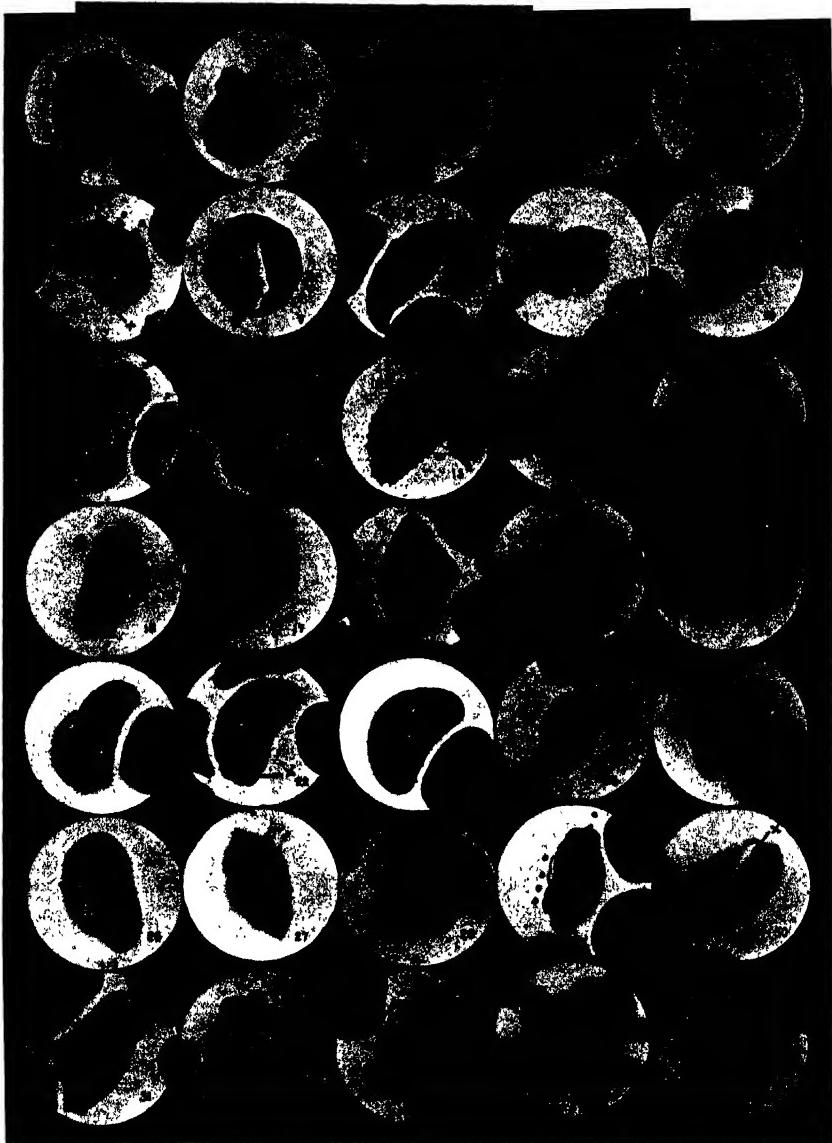
FIG. 49. A pigmented crescent attached to a decolorized red corpuscle. The crescent is bent on itself, thus giving the appearance of a double bib. A hemoglobin mound may be seen at o.

FIGS. 50 to 53. Flagellated crescents. With the exception of Fig. 53, which is attached to a decolorized corpuscle, these crescents are free from corpuscles. They are healthy appearing and the presence of pigment is evidence of previous attachments. The flagella arise from the chromatin substance of the parasites.

Figs. 54 and 55. Free pigmented crescents.

FIG. 56. A very heavily pigmented crescent which, I believe, is in the process of attaching itself to a fresh corpuscle, as the red corpuscle shows no evidence of injury to its hemoglobin. A careful examination of this figure will show the hemoglobin of the red corpuscle extending into the substance of the crescent. The large amount of pigment which the parasite contains is evidence of previous attachments.





(Lawson: *Estivo-autumnal malaria.*)





(Lawson: *Estivo-autumnal malaria.*)



## EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

### III. THE EFFECTS OF A SERUM PRECIPITIN ON ANIMALS OF THE SPECIES FURNISHING THE PRECIPITINOGEN.

By PEYTON ROUS, M.D., GEORGE W. WILSON, M.D., AND JEAN OLIVER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 12, 1919.)

It has been shown previously that specific antisera for infections of which the exciting agent is unknown can be produced in some instances by the use of infected tissue itself as antigen; and that when such sera have been detoxified they can be used successfully for therapeutic purposes.<sup>1</sup> The toxicity is due to the presence of antibodies elicited by the tissue component of the antigen, which, needless to say, are highly injurious to animals of the species furnishing said antigen. It is easy to remove the more obvious of these injurious elements—hemolysins and hemagglutinins—by their selective absorption with red corpuscles, as was done in our type experiments. The precipitins we then encountered were weak and without recognizable action on the animal organism. Whether strong precipitins can cause damage has remained for determination, as has the point of how such damage could be avoided. The problem is not without practical significance in its relation to the utilization of the serum of infected human individuals as antigen.

The study of anaphylaxis has led to the development of a large and complex literature on the effects of the union of precipitin and precipitinogen upon an animal organism to which both are foreign; and a number of papers have been published on the results of injecting precipitinogen into animals which have developed a circulating precipitin. But there exist few observations, and these more or less casual, regarding the effects of a precipitin upon animals against

---

<sup>1</sup> Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 283.

whose serum it is specifically effective. This alone concerns us here. Uhlenhuth and Haendel<sup>2</sup> state in a foot-note to other matters that guinea pigs show severe, anaphylaxis-like symptoms after the intraperitoneal injection of 0.5 to 1 cc. of an anti-guinea-pig rabbit serum with a precipitin titer of 1 to 20,000. The same serum in amounts of 0.75 to 2 cc. caused death. They give a few protocols suggesting tolerance to a second injection. Doerr and Moldovan<sup>3</sup> made closer observations. They state that the precipitating anti-guinea-pig rabbit serum which they employed had *in vitro* a slight hemolytic activity for guinea pig cells, no more than that of normal rabbit serum, yet 2 cc. intravenously killed guinea pigs within 2 hours, while 1 cc. gave rise to severe symptoms, and 0.5 cc. to dyspnea and slight symptoms. Following an intraperitoneal injection there was heightened resistance to a second injection 24 hours later. The experiments cited are few; and the authors make no mention of the presence or absence of hemagglutinins, nor, it seems, did they look for evidence of *in vitro* hemolysis. These are important points for the interpretation of their work, as will be shown.

#### *Removal of Hemolysins and Hemagglutinins.*

It is well known that immunization with blood serum as antigen leads to the development not only of precipitins but of hemolysins and hemagglutinins, even though care has been taken to render the serum free of formed elements. In our first experiments along the lines of the work just described it became evident that these antibodies were seriously to be reckoned with. Hemolysis was never noteworthy on *in vitro* tests, being practically absent when guinea pig complement was employed, and in this regard our sera corresponded with the serum of Doerr and Moldovan. Yet these same sera, inactivated, gave rise *intra vasam* to a profuse breaking down of red cells, as evidenced by hemoglobinuria, hemoglobinemia, extreme anemia, spodogenous spleen, and free blood pigment in the fluid of the body cavities. Some fatalities were manifestly attributable to this cell destruction combined with hemagglutination. The latter phenomenon was always pronounced both *in vitro* and *in vivo*. That death can be due to it alone is well recognized. The blood of the animals sometimes showed an almost massive clumping. In view of all these findings the fact that the precipitating sera gave rise, as they did, to the more or less sudden death of guinea pigs was not surprising.

<sup>2</sup> Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1909-10, iv, 761.

<sup>3</sup> Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

In order to free the sera of hemolysins and hemagglutinins resort was now had to selective absorption with guinea pig red cells. Our technique for this has been described in a previous paper.<sup>1</sup> Care was taken to keep the serum sterile and to free it of all possible stroma fragments by prolonged centrifugation prior to injection. Also, it was overabsorbed, that is, exposed to far more guinea pig red cells than were enough to remove the demonstrable antibodies for these elements. Our experience,<sup>1</sup> like that of others, has been that the strong hemolytic and hemagglutinative serum resulting from immunization with red cells is completely deprived of toxicity when thus treated. Such was far from being the case with the precipitating serum now in question. After absorption it retained the major part of its toxicity, giving rise to sudden death almost as often, in almost the same dose, and with the same symptoms as when untreated save for inactivation. But there was the difference that all lesions referable to hemolysis and hemagglutination were now lacking. Thus a number of late fatalities were avoided. Manifestly, from these results, Doerr and Moldovan were correct in supposing that the toxicity of their serum was due to another element than hemolysis.

#### *Removal of the Precipitin Does Not Remove Toxicity.*

What is this other element? The guinea pig cells used in the absorptions were freed of serum by careful and repeated washing in "gelatin-Locke's" solution, which keeps these usually fragile cells intact. The rabbit serum repeatedly incubated with several successive portions of them remained clear and almost free from hemoglobin. Friedemann<sup>4</sup> has shown that mixtures of red cells and hemolysin in the presence of complement may yield a toxic body before any hemolysis occurs. But this does not happen when complement is absent, as was regularly the case in our work. The possible influence of traces of the washing solution can be ruled out on the basis of previous experience. It seemed likely that the precipitin content of the serum, which was not lessened by repeated absorptions, constituted the toxic element. And in line with this idea, though not necessarily evidence for it, was the fact that the toxicity of different serum specimens

<sup>4</sup> Friedemann, U., *Z. Immunitätsforsch., Orig.*, 1909, ii, 591.

varied in general with their precipitin titer. Attempts were made, therefore, to detoxify the sera by the removal of their precipitin content through specific precipitation. The precipitin was readily removed. But to our great surprise the sera remained as toxic as before.

### *Specimen Experiments.*

*Experiment 1.*—Two rabbits which had received five intraperitoneal injections of guinea pig serum at intervals of 6 days were bled to death from the heart 10 days after the last, and the serum was at once pooled, inactivated, and tested for hemolysin and hemagglutinin. The undiluted serum caused a faint trace of hemolysis when incubated for 2 hours with equal parts of a 5 per cent suspension of guinea pig red cells and a 1 in 10 dilution of fresh guinea pig serum. In such mixtures agglutination was massive with quarter strength serum and was faintly seen with a 1 in 32 dilution of it. The injection of 2 cc. of the serum into the ear vein of a 200 gm. guinea pig<sup>5</sup> was followed in a few minutes by sneezing, restlessness, severe dyspnea, and complete prostration, with slow recovery during the next 12 hours.<sup>6</sup> The urine for some hours after the injection contained much hemoglobin.

50 cc. of the inactivated serum was now incubated under aseptic conditions with four successive portions of guinea pig red cells, twice washed in a large excess of gelatin-Locke's solution. The portions consisted of 7.5, 7.5, 9, and 6 cc. of packed cells respectively, and the period of incubation ranged from  $\frac{1}{2}$  to 3 hours. No agglutination was observable in the last two serum-cell mixtures, and *in vitro* tests showed the complete absence of hemolysin. The absorbed serum, when injected into two guinea pigs of 225 and 215 gm. weight, in amounts of 2 and 1.35 cc. respectively, gave rise to exactly the same symptoms as the unab-sorbed, though they were somewhat less severe. The urines of both animals remained free from hemoglobin.

The precipitin titer of the serum was now taken in mixtures of a constant amount of antibody with decreasing antigen, so as to avoid solution of the precipitate in an excess of the latter. By the use of a blood-counting pipette, previously standardized with mercury, as a measuring chamber, small amounts of the undiluted sera were mixed as such. Precipitation occurred in mixtures up

<sup>5</sup> For the technique of such injections see Rous, P., *J. Exp. Med.*, 1918, xxvii, 459. The operation is rendered more simple and certain if the ear is fixed on a ground glass platform, instead of the opaque one previously described, and trans-illumination is employed.

<sup>6</sup> 3 to 4 cc. of normal rabbit serum can be injected into the circulation of a 200 to 250 gm. guinea pig without the production of symptoms (see Friedberger, E., *Med. Klin.*, 1910, vi, 510).

to and including that containing 5,120 parts of rabbit serum to 1 from the guinea pig, and in 20,000 to 1 when the antigen was diluted to a constant bulk with salt solution after the usual method. The greatest precipitate, coarsely floccular, was seen at approximately 80 to 1 of the whole sera. Accordingly the bulk of the remaining rabbit serum was mixed with sterile guinea pig serum in this proportion, incubated 2 hours, left in the cold over night, and centrifuged until free from the several cubic centimeters of precipitate. It now failed to cause any clouding in mixtures with guinea pig serum above 16 to 1, yielded a slight cloud at 8 to 1, a dubious trace at 4 to 1, and none at 1 to 1. Yet this same serum, injected intravenously into three guinea pigs of 225, 205, and 225 gm. in amounts of 2.2, 1.45, and 1.35 cc. respectively, killed the first animal in 3 hours and 8 minutes, the second in 5½ hours, and in the third gave rise to a moderate "shock" with symptoms resembling those of anaphylaxis.

The results suggest that the serum was rendered, if anything, more toxic by the repeated absorptions.

It is known that a slight, slow precipitation takes place in pooled precipitin sera from different individuals of the same species; and Friedberger<sup>7</sup> has shown that precipitating mixtures will, in the presence of complement, yield a toxic product *in vitro*. To rule out this possible factor in the results, our tests were repeated with individual rabbit serum; and when several sera were to be pooled they were often subjected beforehand to a separate inactivation. In neither case was any difference noted in the results. In the experiment which now follows the sera were inactivated immediately after pooling, and subjected to precipitation with guinea pig serum prior to the absorptions with red cells.

**Experiment 2.**—The sera of four precipitin rabbits were pooled, inactivated, the precipitin titer was taken, whole guinea pig serum being used, and on the basis of the findings most of the pooled serum was submitted to an optimum precipitation. This was all done on the same day, as rapidly as possible. The optimum precipitation occurred in a 90 to 1 mixture with undiluted guinea pig serum, but clouding was noted in mixtures up to and including 5,120 to 1. The untreated serum injected intravenously into two guinea pigs of 325 and 400 gm., in amounts of 1.65 and 1.8 cc. respectively, killed the first mentioned animal within ¼ hour and caused great, though brief, prostration of the second. 1.85 cc. of the treated serum killed a 275 gm. guinea pig in 1 hour, and 2 cc. caused moderate symptoms in a guinea pig of 375 gm. All four animals had hemoglobinuria.

<sup>7</sup> Friedberger, E., *Z. Immunitätsforsch.*, Orig., 1909–10, iv, 636.

Both the treated and untreated sera were now submitted to five successive absorptions with twice washed red cells in the proportion of 23 cc. of serum to 2.3, 2.3, 2.3, 4.05, and 3.8 cc. of packed red cells. The contact periods ranged from 1 to 2 hours. Agglutination, which in the first mixture was well marked, diminished to a trace in the last one—an exactly similar trace for both the "precipitated" and untreated serum, as *in vitro* tests showed. No hemolysin could be found in the test-tube, with guinea pig complement. 1 part of salt solution was now added to 90 of the unprecipitated specimen, and comparative intravenous injections were carried out with it and with the precipitated serum (Table I).

TABLE I.

Serum absorbed only.			Serum absorbed and precipitated.		
Weight of animal.	Amount injected.	Result.	Weight of animal.	Amount injected.	Result.
gm. 275 275	cc. 2.1 2.0	Died in 1 hr., 51 min. " " 1 " 5 "	gm. 250 275 275	cc. 2.1 1.8 2.2	No symptoms. " " Very severe shock.

Tests showed that the absorbed and precipitated serum still contained enough precipitin to cause clouding in mixtures up to and including 320 to 1 with whole guinea pig serum. An optimum was found at 20 to 1 and the serum submitted to a new precipitation in this proportion. Thereafter it still gave a moderate flocculation with an equal amount of guinea pig serum, but no clouding in mixtures above 10 to 1, or 16 to 1 when the precipitinogen was diluted with salt solution. Comparative tests *in vivo* were again made. 1 part of salt solution was added to 20 parts of the unprecipitated serum prior to the injections (Table II).

TABLE II.

Serum absorbed only.			Serum absorbed and precipitated.		
Weight of animal.	Amount injected.	Result.	Weight of animal.	Amount injected.	Result.
gm. 375 300 300 275	cc. 2.0 1.8 2.15 2.2	Moderate shock. Severe " Died in 11 min. Severe shock.	gm. 300 250	cc. 2.2 2.2	No symptoms. Very severe shock.

At every stage in the treatment of the serum, cultures on agar and in bouillon were taken. These remained uniformly sterile.

In this instance, in contrast with Experiment 1, the attempts to detoxify the serum seemed to have some degree of success, and we were encouraged to further trials. These will not be detailed. They showed that individual differences in the test animals were mainly accountable for the wide variations in the results. Some animals were practically unaffected by the serum that killed others; and no matter how thoroughly the serum was exhausted with red cells and freed of precipitin it remained highly injurious. This was true even when it was given locally. For when injected subcutaneously or into the skeletal muscle it produced a severe lesion. But before describing this its general effects will be taken up.

#### *Effects of the Serum Deprived of Precipitin.*

In guinea pigs reacting to an intravenous injection of the exhausted and precipitated—or, for that matter, unprecipitated—serum there is a latent period of from 3 to 10 minutes during which the behavior is normal. Then the animal becomes restless, running about, scratching itself, perhaps sneezing, springing into the air, or twitching. The hair roughens, the urine and feces are usually voided, and an inspiratory dyspnea rapidly appears, accompanied in severe instances by cyanosis and complete prostration, followed by death in a few minutes or hours. Occasionally convulsions precede the fatal issue. Often there is only a paresis of the hind legs, or the animal is now prostrate, now on its feet again, and in these instances of milder symptoms recovery may be very rapid. More often, while recovering, the guinea pig sits crouched, cold, and with staring coat for some hours. When handled it is passive and weak. But by the next day recovery seems complete, and further observation proves that it indeed is so.

The animals that succumb show little on gross examination. The lungs may be distended, and the blood fail more or less markedly to clot, as in anaphylaxis; but these are by no means constant findings. There may be fresh petechiae in the lungs and intestinal mucosa, as so often after violent death of any sort. This is all that is found if the animal has died within a few minutes of the injection. When it has survived for some hours the liver is always greatly congested, and in it there may be observed microscopically the only lesion that is

*Relation of the Phenomena to Anaphylaxis*

The "shock" produced by the serum will be seen from our description to resemble strikingly in both guinea pigs and dogs that called anaphylactic; but on close analysis points of difference declare themselves. At autopsy the lungs of guinea pigs may not be found distended, though they often are so; and the blood may be clotted. The latent period after an intravenous injection is much longer in dogs than that preceding the anaphylactic paroxysm. More important is the fact that desensitization cannot be effected either by small, graduated injections or by one that results in shock. We have given especial attention to this point, since Doerr and Moldovan present a few protocols which seem to indicate that they succeeded in desensitizing with their precipitating serum, and if this were the case it would offer a way to the safe, therapeutic utilization of our own. But their results must be referable to individual animal variation such as has already been mentioned, for our many tests have definitely shown that even after an injection of absorbed, or absorbed and precipitated, serum which calls forth a severe reaction, there may be no tolerance whatever to a second dose, whether it be given into the blood stream or locally. This is true in dogs as well as in guinea pigs. For example, a dog weighing 4 kilos was given 11.6 cc. of exhausted and precipitated serum into an ear vein. There resulted severe "shock," but with rapid and apparently complete recovery, 4 days later another and similar injection was given, and this called forth exactly the same severe but transient reaction. The animal was killed 6 days after the second injection, and in its liver active repair was found to be taking place of a recent hemorrhagic lesion such as has already been described. When several, small, desensitizing doses or one large one were used (in guinea pigs) the results were no better. The local effect in the guinea pig has some similarity to the Arthus phenomenon, but the latter is in our experience a less severe type of lesion in this species and far more difficult to elicit, at least with horse serum, while it lacks the hemorrhages caused by the absorbed and precipitated rabbit serum.

It is well known that the development of serum sickness is accompanied in man by the appearance of precipitins in the blood, and

that the urine may show a coincident albuminuria. We have followed the urines of a number of dogs and guinea pigs subjected to severe "shock" by the intravenous injection of precipitated or unprecipitated serum which had been exhausted with red cells. None showed noteworthy urinary change. Casts were regularly absent, and the slight trace of albumin occasionally noted was no greater than was inconstantly present prior to the injection.

Despite all this, there is no denying that the effects of the serum may be due to the same toxic principle or principles concerned in anaphylaxis. But, if so, an important difference in the quantitative relations must be assumed.

#### *Possible Sources of the Toxicity.*

Is the toxic element primarily present in the serum, or is it engendered by treatment? We feel convinced that the former is the case. The "shock" produced by the absorbed, or absorbed and precipitated, serum differs in no essential from that caused by inactivated but otherwise untreated precipitating serum, as observed by Uhlenhuth and Haendel, Doerr and Moldovan, and ourselves. Were it due to hemolysins and hemagglutinins persisting after absorption, the examination of the blood and the autopsy findings would give evidence of this, while furthermore the process of repeated absorption, even if incomplete, would greatly diminish the toxicity of the serum. Neither is the case. Our serum, as already mentioned, was over-absorbed; that is, submitted to more red cells than were necessary to take out all demonstrable hemolysins and agglutinins, a process which renders completely innocuous the ordinary hemolytic and hemagglutinative serum.

Many observations were made which bore on the question of whether gross precipitation within the animal body might not be a cause of the disturbances noted. Always the precipitated serum failed *in vitro* to cause a clouding when mixed with even a little more than its bulk of guinea pig serum, far less than the preponderant quantity encountered on its injection into the blood. Such tests would seem to rule out actual precipitation as the cause of disturbance, unless indeed the conditions with plasma differ greatly from

those with serum—and there is no reason to suppose that they do, since complement is unnecessary for the precipitin reaction. We have said that the toxicity of the different sera varied in general with their original precipitin titer. Yet that the "shock" engendered by the serum had no essential dependence on the immediate precipitin content was well shown in the results with serum from which the precipitin had been removed. The possibility remains that the toxic element may be a product of the interaction of precipitin and precipitinogen, one formed as readily when the two are brought together without as within the animal body. Against this is the fact shown by Friedberger<sup>7</sup> that specific precipitation *in vitro* fails to give rise to a toxic element unless complement be present; for it was absent in our experiments. But Friedberger made his tests of toxicity on animals of a species to which both precipitin and precipitinogen were strange, whereas in our work the precipitin was directed against the serum of animals of the species used for the tests, and just such serum was employed for the *in vitro* removal of precipitin.

There remains the interesting possibility of the presence in the serum of a hitherto unrecognized toxic antibody. Further work alone can justify any speculation in this direction.

#### SUMMARY.

There is present in serum of high precipitin titer, produced by the repeated injection of rabbits with the blood-free serum of guinea pigs or dogs, a principle highly toxic for animals of the species furnishing the antigen. Intravenously the serum causes severe shock, and even sudden death, while locally it gives rise to acute inflammatory changes and profuse capillary hemorrhages. The complete removal of hemolysins and hemagglutinins from the serum by exposing it repeatedly to washed red cells lessens its toxicity to only a slight degree and one obviously dependent on these elements; while the further removal of precipitin by specific precipitation *in vitro* has no detoxifying effect whatever. Whether the toxic principle is a hitherto unrecognized antibody or perhaps a toxic product of the interaction of precipitin and precipitinogen,—one formed as readily in the test-tube as in the animal body,—remains to be determined.

The symptoms of guinea pigs and dogs given an intravenous injection of treated or untreated serum markedly resemble those of anaphylaxis, but our attempts at desensitization have been unsuccessful. The local lesion in guinea pigs is more severe than that of the Arthus phenomenon. But these differences from anaphylaxis may, of course, be dependent merely on differing proportions of constituents that are themselves, as yet, scarcely apprehended.

Our observations, as here summed up, were made with a practical point in mind, and as regards this point they are of a discouraging nature. In papers already published it has been shown that sera specifically effective against infections of which the excitant is unknown can in some cases be obtained by using infected tissue itself as antigen. Such sera must, of course, be deprived of antibodies injurious to tissue, prior to their employment in the animal body; and this was successfully accomplished in our early experiments by exhaustion with washed red cells. The purpose of the present work was to determine whether serum used as antigen gives rise to injurious principles in the antiserum. For the serum of infected individuals would in many diseases form a convenient antigen. It is evident that injurious principles result from its use, and that they are not removed from the antiserum when the latter is exhausted with red cells and its precipitin removed by specific precipitation, nor can their action be nullified by desensitization as carried out in anaphylaxis. Unless the obstacle of their presence is in some way overcome the body fluids of infected human beings cannot be practically utilized for the production of antiserum. In test animals the difficulty is not so grave. For we have found that the toxic antiserum produces no enduring lesions when it is administered intravenously in non-lethal doses.



## REGENERATION AND NEOTENY.

By EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 29, 1920.)

According to Kamerer, it is possible to prevent metamorphosis in the caudate amphibians by amputation of the legs or tails of the larvæ. In my attempts to produce neoteny in the larvæ of salamanders, however, Kammerer's method has been tried without success, my experiments failing completely to confirm Kammerer's positive statements with regard to the effectiveness of the method.

Dumeril<sup>1</sup> observed that among several larvæ of *Ambystoma tigrinum*, which were the offspring of neotenous animals (axolotls), only those metamorphosed which had been deprived by their comrades of legs and part of their tails. He believed, therefore, in contradistinction to Kammerer, that regeneration may induce metamorphosis in larvæ which without regeneration would become neotenous. Later, however, he observed that larvæ of the same lot, which had not been mutilated, also metamorphosed; hence he was finally convinced that regeneration had nothing to do with metamorphosis of the larvæ.

Kammerer<sup>2</sup> experimented not only on the larvæ of caudate amphibians (*Triton cristatus* and *Triton alpestris*), but also on those of *Salientia*. He amputated the limbs as well as the tail. He concluded from his experiments that

"Injuries of any sort effect metamorphosis in directly opposite ways in Urodela and Anura as demonstrated with greatest certainty in the experiments. While in the salamander larvæ neoteny is brought about without the slightest difficulty, if only one limb or a piece of the tail is removed, the same procedure induces a rapid appearance of the transformation symptoms in the tadpole."<sup>3</sup>

---

<sup>1</sup> Dumeril, A., *Ann. Sc. Nat. Zool.*, 1867, vii, 229.

<sup>2</sup> Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1905, xix, 148.

<sup>3</sup> Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1905, xix, 176.

Though such a difference between two different groups of animals is possible, it does not seem likely that metamorphosis should be controlled in different groups of amphibians by mechanisms so different; at least such an assumption does not seem justified by the evidence presented in Kammerer's paper. In the first place, that which Kammerer calls neoteny in his *Triton* larvæ is, as far as his recorded data are concerned, only a difference of 1 month. In one instance the operated animals transformed 1 month later than the controls; in the second experiment the operated larvæ had not metamorphosed 14 days after the controls had completed metamorphosis; at this time the experiments were discontinued.<sup>4</sup> Nowhere do we find any indication that the sex organs were actually examined to make sure that they had developed at a more rapid rate than the rest of the organism. Nor do we find any proof that these small differences had not been produced merely by differences in the quantity of food or that they were not due to the fact that the larvæ of the different sets were the offspring of different females. Since the same objections could be raised with regard to his experiments on tadpoles, it becomes doubtful not only that so fundamental a difference exists between *Caudata* and *Salientia* as that claimed by him, but also whether amputation and regeneration had any effect on the metamorphosis of Kammerer's larvæ at all. In a later article<sup>5</sup> he emphasized the fact that the retardation of metamorphosis in his larvæ was not due to a retardation of growth because of insufficient food; he says:

"Individuals particularly suited for the production of the phenomena of neoteny are those which have been subjected to experiments on regeneration, since they as a rule retain . . . for a long time after the removed parts have been replaced the larval condition without showing any particular inhibition of the general growth of the body; hence they turn into truly neotenous, not into starved larvæ!"<sup>6</sup>

It is in this case of course extremely difficult to form any opinion about the causes which lead to retarded metamorphosis, since apparently these animals were well fed, but it is well known, and we have

<sup>4</sup> Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1904, xvii, Experiment XI, p. 167, and Experiment XII, p. 168.

<sup>5</sup> Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1904, xvii, 165.

<sup>6</sup> Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1904, xvii, 240.

discussed it in a previous article,<sup>7</sup> that the larvæ of all species so far examined must grow longer in low temperature than in high temperature before metamorphosis can take place, without, however, under these circumstances becoming neotenous. Unfortunately Kammerer does not mention at what temperatures his larvæ were kept. In the same article Kammerer refers to neotenous larvæ of *Salamandra maculosa*, 2 years old; it is very probable that these larvæ were truly neotenous. The experiments reported in this paper make it, however, very doubtful that this result could have been obtained by merely cutting off the limbs and the tails. I tested the effect of the removal of the limbs and of the tail followed by regeneration of these parts first in the species *Ambystoma opacum*. In the fall of 1916 the eggs of one female were collected and twenty-eight of them divided into four series; two series (E and G), consisting of six larvæ each, were used as experimental series, E being kept at approximately 25°C., G at approximately 15°C.; each experimental series was controlled by a series consisting of eight animals (A and C). The larvæ were measured and examined at least once every week. They were kept in individual jars, and individual records were made. Both fore limbs were removed from the larvæ of Series E and G 46 days after hatching, and 88 days after hatching 50 per cent of the tail was cut off. In Series E the regeneration of the legs was nearly completed 102 days after hatching, *i.e.* 80 days before metamorphosis; in Series G the legs had not regenerated to their normal length 109 days after hatching, but from this time on regeneration occurred at a very slow rate, and the animals never possessed legs of normal length. Regeneration of the tails was most vigorous during the first weeks after they had been cut off but continued in both series throughout the larval period.

Table I shows the result of this experiment. For reasons discussed in other papers,<sup>7</sup> metamorphosis was regarded as taking place at the time when the first molt occurred; consequently the figures recorded in this table represent the number of days after which the animals shed their skins for the first time. In several larvæ this figure was not recorded, the date when they were set on land being recorded instead. At 25°C. this was done on the same day as the first molt, or 1 day

<sup>7</sup> Uhlenhuth, E., *J. Gen. Physiol.*, 1918-19, i, 525.

later, and the error caused by this difference is very small. At 15°C. in only one instance was the day of the first molting not recorded, and the error in this instance may amount to from 1 to 4 days. As the figures show, there was no difference between the controls and the experimentals at 15°C., both series metamorphosing 243 days

TABLE I.

*Regeneration and Metamorphosis in Ambystoma opacum.*

All four series fed on earthworms; A and E kept at approximately 25°C. average temperature, C and G approximately 15°C. average temperature. In E and G, at 46 days after hatching, both fore limbs were cut off, and at 88 days 50 per cent of the tails was cut off.

No.	25°C.		15°C.	
	A Control.	E Regenerating.	C Control.	G Regenerating.
	days	days	days	days
1	191(Land.)	216(Land.)		236
2	200	206	241	235
3	169(Land.)	182(Land.)	249	+
4	184	+	234	239
5	186	209(Land.)	247	+
6	+	199	+	261(Land.)
7	172		242	
8	199		+	
Average.....	186	202	243	243
Difference.....		16		0

after hatching. At 25°C. the difference was very small; the regenerating series metamorphosed only 16 days later than the controls.

These results show that in *Ambystoma opacum* neoteny cannot be produced by removal of parts of the body and their regeneration. Beyond this no conclusions can be safely drawn. It should be mentioned, however, that in all four series approximately the same amount of food was available for the animals. The earthworms which served as food were given in pieces of approximately the same size. Since, however, the amount of food in these series was not large enough to cover the demand of normal growth, the animals were partly starved, as may be seen also from the length of the larval period, which amounts

to 186 days in Series A, while it is only 60 to 100 days at 25°C. if an unlimited supply of food is allowed the larvæ. For this reason it is possible that at high temperature (25°C.), at which regeneration proceeds at a more rapid rate than in lower temperatures, the regenerating larvæ were less well supplied with food than the controls, notwithstanding that both received an equal amount, and that this circum-

TABLE II.

*Regeneration and Metamorphosis in Ambystoma tigrinum.*

Both series kept at 15°C. In Series LVI the fore legs were removed at 26 days, from 4.5 to 11 cm. of the tails were removed at 47, 61, 68, 82, 96, 110, 124, and 138 days, and the hind legs were removed at 61 days.

No.	XLVIII Control.	LVI Regenerating.
1	days	days
2	123	124
3	131	145
4	145	148
5	122	130
6	120	148
6	137	122
Average.....	130	136

stance caused the delay in metamorphosis in Series E. The experiments were apparently unsatisfactory also because they did not warrant a generalization of the conclusion that neoteny could not be brought about by removal of the parts of the body, since the species used might be less prone to neoteny than *Triton* and *Salamandra maculosa*.

Accordingly the experiment was repeated in the spring of 1919 with the larvæ of *Ambystoma tigrinum*, a species frequently found in neotenous condition. With respect to food, a more satisfactory condition was established by placing in the jars every day an amount of earthworms greater than was required by the larvæ. Two series, a regenerating (LVI) and a control (XLVIII), were kept at 15°C.; both were the offspring of the same female, and each consisted of six animals. They were kept in separate jars and the observations

recorded individually. In Series LVI the fore limbs were removed at 26 days, the hind limbs at 61 days, and 11 cm. of the tail at 47 days after hatching. To assure continuous regeneration the tails were clipped as soon as part of the previously removed tips had regenerated (at 47, 61, 68, 82, 96, 110, and 124 days after hatching).

The result is summarized in Table II. It was practically the same as that of the first experiment. The larvæ of Series LVI metamorphosed (*i.e.* shed the skin for the first time) 6 days later than the controls. Since this difference is smaller than the differences observed among the larvæ of the same series it may be said that in both series the larvæ metamorphosed at the same time. And certainly there was no neoteny produced by removal of even considerable amounts of tissue (42.4 mm. of tail were removed by the successive clippings of the whole larval period), though the species used is one which would be expected to yield readily to influences producing neoteny in a species like *Salamandra maculosa*, which only rarely is found in neotenous condition.

#### CONCLUSIONS.

It is apparently quite certain that removal of parts of the body (limbs, tail) followed by regeneration of these parts (1) does not produce neoteny in the larvæ of salamanders, and (2) has no influence upon metamorphosis.

[Reprinted from THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, March 27, 1920, Vol. lxxiv, No. 13, pp. 865-869; and the TRANSACTIONS OF THE ASSOCIATION OF AMERICAN PHYSICIANS, 1919, Vol. xxxiv, pp. 193-205.]

## LETHARGIC ENCEPHALITIS.

### HISTORY, PATHOLOGIC AND CLINICAL FEATURES, AND EPIDEMIOLOGY IN BRIEF.

By SIMON FLEXNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

It is of more than ordinary interest that within the last dozen years at least two epidemic diseases, affecting chiefly the central nervous system, have prevailed widely in Europe and America. The first is poliomyelitis, which since 1907 has passed through several phases in the United States, and culminated in the outbreak of unparalleled severity, centering in New York State, in 1916. The second is lethargic encephalitis, apparently only recently introduced in and already widely distributed through this country. It is highly desirable that the main facts known about the latter disease should be given publicity; and it may well be that the experience, often of tragic nature, gained with poliomyelitis, may serve us in dealing more effectively with the encephalitis peril. Hence, by way of introduction, I shall sketch the recent history of poliomyelitis.

#### *Poliomyelitis.*

The occurrence of poliomyelitis in America and generally in Europe beginning in 1907 followed, it now appears, the severe epidemics of Norway and Sweden, which reached a first climax in 1905. Indeed, poliomyelitis became so widespread after 1905 that it may be regarded as having become pandemic. Besides Europe and the United States, South America, Australasia and the far eastern countries all were involved. Poliomyelitis, it is true, had been mildly epidemic in the United States before 1907; but the most severe of the earlier outbreaks were inconspicuous as compared with the later ones, both as regards the number of persons affected and the territory invaded. Thus, the severe Vermont epidemic of 1894 included 130 cases; and it is worth noting that no extension into distant territory followed in its wake.

It is customary to ascribe to Wickman, who studied the Swedish epidemic of 1905, and gave to the world in 1907 his remarkable book on epidemic poliomyelitis,<sup>1</sup> the first account of the so-called abortive cases of poliomyelitis. But Caverly, who studied the Vermont outbreak,<sup>2</sup> clearly described cases which did not go on to frank paralysis, but terminated in prompt recovery after symptoms which seemed to presage the onset of paralysis. As these "imperfect" or nonparalytic instances arose along with the frankly paralytic cases, it seemed to Caverly that they belonged in the same category with the latter. This conception is now abundantly justified. And it is due to Wickman that the abortive type of cases of epidemic poliomyelitis is so widely recognized. The epidemiologic significance of the abortive cases is considerable, since many are ambulatory and most are invalidated for a few days at most; they thus furnish a ready means of distributing freely the inciting microbic agent or virus of poliomyelitis in their surroundings. From the public health point of view, the abortive and ambulatory cases of poliomyelitis are more dangerous than the frankly paralytic, since by the very nature of the circumstances the latter are restricted in their contact with healthy persons.

We may think that the forerunners of these epidemics of poliomyelitis were imported into the United States about 1906 or 1907; that the cases of the disease or carriers of the virus first had merely local distribution, but that gradually the conditions favoring a wider spread of the disease became more extensively developed; that thus the more numerous and severer outbreaks arose; until finally, conditions in certain localities having become highly favorable, the terrific explosion with its thousands of paralyzed patients occurred in 1916, since which time the disease, while still present, has reverted to a relatively quiescent state.

The medical profession in general in the United States in 1907 was not informed and ready to deal with the epidemic of poliomyelitis. Practically none of the profession had had previous experience with the disease except in its rare sporadic form, which at first seemed improbably connected etiologically with the epidemic affection. Few

1. Wickman, I.: Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

2. Caverly, C. S.: Med. Rec. 46: 673, 1894.

physicians knew of the succession of outbreaks, small and large, that for many years had been going on in Norway and Sweden. When, therefore, the epidemic appeared in the United States, there was inevitable confusion of diagnosis, lack of understanding of the infectious nature of the disease and the degree of its communicability, uncertainty as to the public health policy to be pursued, and, finally, in places an undue harshness in the endeavor to curtail its ravages.

Gradually a deeper knowledge of the disease by the profession at large has removed many of these disabilities, and a wider understanding of its atypical forms is rendering feasible the early and more complete institution of those protective public health measures with which hope of better control of epidemics is inseparably connected.

#### *Lethargic Encephalitis—Historical.*

With this introduction, I shall now consider certain historical points regarding lethargic encephalitis. It appears that the first cases of that disease recognized in the United States occurred in the winter of 1918-1919. In contradistinction to epidemic poliomyelitis, there is no reason to suppose that this epidemic affection of the central nervous system ever before existed in America. This point is an important one. At present the disease seems to be widely distributed, as cases have been reported from many states.

It is possible to trace the cases of lethargic, or epidemic encephalitis, now arising in this country, to an outbreak which occurred in Vienna and neighboring parts of Austria in the winter of 1916.<sup>3</sup> Because of war conditions, knowledge of this unusual disease did not at once reach western Europe and the United States; but nevertheless cases of the disease occurred in England and France in the early months of 1918, and in America about one year later.

Both in Austria and in England, in which countries the first cases were observed, respectively, in eastern and western Europe, the disease was first mistakenly attributed to food intoxications. In Austria the early cases were ascribed to sausage poisoning, in England to botulism arising from various foods.<sup>4</sup>

3. Von Economo, C.: Die Encephalitis Lethargica, Vienna, 1918.

4. Report of an Enquiry into an Obscure Disease, Encephalitis Lethargica, Report to the Local Government Board, No. 121, London, 1918.

This error is not perhaps as remarkable as might at first sight appear. In the first place, both countries were laboring under unprecedented conditions of food shortage, Austria because of the blockade, England because of the submarine. Moreover, because of this shortage, preserved foods were employed on a scale never before equaled, and, of course, waste and refuse were reduced to a minimum. Furthermore, an early symptom of this encephalitis is third nerve paralysis—giving rise to diplopia, ptosis, etc.—which happens also to be an early symptom in certain forms of food poisoning and notably in botulism.

Ultimately, in both countries the notion of food origin became untenable, and the disease was recognized as arising independently of diet and other usual conditions of life, and came to be viewed as probably of microbic origin and of communicable nature.

#### *Pathologic.*

The first fatal cases, which occurred in Vienna, supplied on histologic study a physical basis for the symptoms observed during life; and the first English and French cases similarly examined microscopically showed lesions identical with those described for the Austrian cases. In due time the anatomic study of cases arising in the United States and still other countries showed close agreement with the others, and now a histologic basis of the pathology of the disease, of remarkable concordance, has been provided. On this basis we may now regard lethargic encephalitis as representing a definite pathologic as well as clinical complex, and to consider it as a distinct disease.

The histologic changes or lesions of lethargic encephalitis may be both extensive and profound. Those so far described as confined to the central nervous system affect particularly the brain and especially the gray matter at the base of that organ. While, indeed, any part of the gray matter may be involved, and lesions are found in the cortex and in the cerebellum, the structures particularly affected are those about the third ventricle, the aqueduct of Sylvius, the lateral ventricle and optic thalamus, and the pons and medulla. The spinal cord is variably involved. In general, it may be stated that the sever-

ity of the cerebral lesions diminishes from before backward; the upper or cervical cord often shows changes; but it is still to be determined how often and to what extent the cord as a whole is affected.

The lesions themselves consist of cellular aggregations about the blood vessels, cellular infiltrations in the nerve tissues themselves, small, often microscopic hemorrhages and an outpouring of plasma or lymph into the tissue interstices (edema). The cellular accumulations and invasions are chiefly mononuclear in nature (lymphocytes, plasma cells, polyblasts); polymorphonuclear cells are also encountered, but are relatively inconspicuous. The lesions themselves occur in nodular and in diffuse forms; and those of the tissues are at times clearly associated with the vascular affections and at other times are so extensive as not to be brought into relation with particular vascular involvements. The paralyses of the ocular, facial and other muscles which sometimes occur arise, with rare exceptions, from the cellular and other invasions of the nuclei of the corresponding nerves.

### *Clinical.*

The clinical phenomena or symptoms of lethargic encephalitis are referable to the lesions of the central nervous organs or the pathologic process, as sketched. It is perhaps too early in the study of the disease to set up hard and fast clinical varieties or types. However, attempts at classification have already been made. One of the most comprehensive is that of MacNalty,<sup>5</sup> which is reproduced here, as it is suggestive and may prove useful in practice. MacNalty distinguishes six groups of cases: (1) cases with general symptoms and without localizing signs; (2) cases with third nerve paralysis and general disturbance in the function of the central nervous system; (3) cases with facial paralysis and general disturbance in the function of the central nervous system; (4) cases with spinal manifestations and general disturbance in the function of the central nervous system; (5) cases with polyneuritic manifestations and general disturbance in the function of the central nervous system; and (6) cases with mild or transient manifestations (so-called "abortive" cases). To these

5. MacNalty, A. S. Report to the Local Government Board, No. 121, London, 1918, p. 12.

should be added cases of paralysis of other motor cranial nerves than the third and seventh such as those of deglutition and respiration.

Probably there is an incubation and prodromal period which precedes the onset of the striking subjective and objective symptoms of the disease; but thus far these have not been defined. Hence the so-called onset of the disease is usually described as sudden or acute. The latter is, indeed, so striking that the patient is able often to tell the precise hour of a particular day on which he fell ill. Actually the striking symptoms often develop more slowly than in poliomyelitis.

The initial symptoms are described as chills, lassitude and general malaise, headache, and general pains, nausea and anorexia, associated often with the common symptoms of upper respiratory catarrhal affections. Fever is an irregular manifestation. It may be present at onset or may appear only later. The temperature range tends not to be high—from 101 to 102—but it sometimes swings to 103 or 104. As the symptoms develop there arise lethargy or drowsiness, vertigo, tinnitus, muscular weakness, blurred or misty vision, diplopia, photophobia, tremors and twitchings, ataxia, delirium, irritability, restlessness, mental depression and other alterations, difficulty in articulation and in swallowing, stiffness of neck and spasticity of other muscles, sweating, hiccup, etc. Among the earliest symptoms to arrest the attention of the patient and the physician are diplopia and ptosis with varying degrees of lethargy. But still other paralyses (e.g., facial) may appear, and the lethargy may arise independently of all localizing nervous signs.

The outstanding feature of the disease is the lethargy, which is progressive in character and present in the great majority of cases (80 per cent.?). It may appear suddenly, but usually is gradual in onset. The patient becomes apathetic and dull, appears dazed or stupid, the hours of sleep become prolonged, and he is hard to wake in the morning. Moreover, he may fall asleep at odd hours—while engaged at work or at mealtime. The lethargy may deepen into stupor or even into coma. Its duration is variable—a week, a month or even longer —up to four months. Even after long periods, recovery may still follow. During the lethargy, there may be lack of facial expression (masklike features). The usual state is one suggesting profound sleep, from which the patient can be aroused by loud speaking, prodding,

etc., to partake of food or answer questions. But cases in which marked restlessness and even mania have been present followed by lethargy have been noted.

Symptoms referable to irritations of the meninges appear. Usually they are slight, and while sometimes arousing suspicion of acute meningitis, that condition is excluded by lumbar puncture and examination of the cerebrospinal fluid. The fluid tends to be under somewhat increased pressure, but clear. The number of cells is slightly increased (very rarely 100 per cubic millimeter), and the globulin content little and sometimes not at all excessive. The cells, which range usually around 10 to 20 per cubic millimeter, consist partly of mononuclear and partly of polymorphonuclear leukocytes. In addition, the important point of the rare presence of Kernig's sign should be mentioned.

The occurrence of paralyses of the face muscles has been mentioned. Paralysis of the extremities is rare: wrist drop has been noted in at least one instance. But a far more common symptom is rigidity or spasticity, chiefly of the extremities, which in a few cases has been observed to extend to the spinal and even the facial muscles, making a picture suggestive of paralysis agitans. This spasticity of the extremities is ascribable to involvement in the encephalitic process of the lenticular nucleus and the corpus striatum.

The duration of the stupor is very variable; it may last a few days, for weeks or even for months, and recovery still take place. The return to clear mentality is usually gradual; muscular power also tends to return slowly, and general convalescence tends to be prolonged. In paralytic examples of the disease, rapid, complete or partial clearing of the palsies has been noted.

The number of cases of undoubtedly lethargic encephalitis thus far reported is too small to indicate the age periods of greatest incidence. For the present it may be stated that the disease occurs at all ages, namely, from a few months to advanced years (over 70 years). Likewise, it appears as if the two sexes were about equally attacked.

The fatalities reported range from 20 to 35 or 40 per cent. Probably the higher mortalities refer to groups of the severer cases of the disease. Since knowledge of the disease is still very restricted and diagnosis still in its beginnings, probably many cases of lighter affec-

tion are overlooked or given other names and interpretations, thus making it impossible at present to arrive at an accurate estimation of the prognosis and mortality. At best, however, the disease is to be regarded as serious, whether from the point of view of long duration from onset to restoration to health, or of fatality. The chief immediate causes of death reported have been intercurrent pneumonia and paralysis of the respiratory center in the medulla.

Present indications are that the degree of communicability of lethargic encephalitis or susceptibility to the disease is low, possibly equaling that of epidemic poliomyelitis as observed in ordinary times. The seasonal incidence seems to be midwinter, in that respect resembling epidemic meningitis and differing widely from epidemic poliomyelitis, which prevails usually in midsummer and early autumn.

Explanations of the lethargic state have been offered. A toxic origin is, of course, possible. It seems more likely, however, in view of the nature and distribution of the lesions, that its source is rather a mechanical one. It is known that the sensory stimuli from the special and other senses pass by way of the thalamus to the cerebral cortex.<sup>6</sup> Since, therefore, the thalamus is so commonly the seat of the cellular infiltrative lesions described, it would appear that the stimuli are interrupted in that organ on the way to the cortex, whence a kind of sleep supervenes. The obstruction to the stimuli is not absolute, since the patient can be aroused by increasing their intensity (as by loud speaking, prodding, etc.).

#### *Epidemiologic.*

It is now sufficiently obvious why the popular name of "sleeping sickness" has been applied to this malady. The disease is, of course, wholly distinct from African sleeping sickness, which is a trypanosomal infection carried from person to person by means of an insect vector—the tsetse fly.

When an apparently new disease arises, it is always important to inquire whether the particular set of symptoms that are taken to characterize it has been observed and recorded before.

6. Head, H., and Holmes, G.: Brain 34: 102, 1911-1912.

In the present instance there are two significant records which may easily refer to a similar and possibly identical disease. The first one dates from 1712 and refers to an outbreak of so-called sleeping sickness centering about Tübingen in Germany. The second record dates from 1890 and deals with a rather puzzling malady called *nona*, which is described rather in the lay than the medical literature of the time and seems to have prevailed in the territory bounded by Austria, Italy and Switzerland. In respect to neither instance, however, do the records contain the minuter data which would admit of a certain identification of the disease described with the encephalitic malady we are considering.

One circumstance is, however, significantly suggestive. The location of the 1890 affection "*nona*," which was characterized by somnolence, stupor and coma, coincides roughly at least with that of the first cases reported in the present epidemic. The question may, therefore, well be raised whether the endemic home of this epidemic variety of encephalitis may not be that corner of southeastern Europe overlapping the three countries mentioned. If this should prove to be probable, the next questions to arise would relate to the circumstances under which the disease slumbered on in ordinary times, and to the conditions that favored a greater activity and a wider spread about the year 1916.

To deal with the first one will require particular and intensive studies carried out with the especial object in view to disclose hidden cases in the region originally affected. An answer can in the meantime be hazarded to the second question. The depressing effects of war, acting by way of hunger, cold, migrations of populations, and general insanitation, might initiate the conditions through which a low endemic might well be converted into a higher epidemic incidence of the disease.

In effect, a similar set of depressing and favoring conditions may be supplied by a highly debilitating and destructive epidemic, such as the periodic waves of pandemic influenza which recur from time to time. In this manner may possibly be explained the coincidence

7. The etymology of this term is not known. It has been suggested that it is merely a corruption of the term "coma."

of the Tübingen epidemic of 1712, also called sleeping sickness, and of the "nona" of 1890 with epidemic influenza, just as the wider distribution of the encephalitic malady and the influenza epidemics of 1918 and of 1920 may be similarly associated. In other words, what the depressing circumstances of the war did for Austria-Hungary in 1916, the pandemic of influenza may have done for the rest of the world in 1918 and subsequently, namely, prepare the soil, as it were, for the growth in number of cases and for increase in intensity and capacity for spread of an infectious nervous disease ordinarily narrowly localized and moderately benign.

This relationship of lethargic encephalitis to the epidemic of influenza has, indeed, led to a discussion as to whether the former is not merely a sequel—early or late—attending a certain, if only small, number of cases of epidemic influenza.

Regarded merely chronologically, the question thus presents itself: In 1916, when the first cases of encephalitis appeared or at least were recognized in Austria, the epidemic of influenza which prevailed later, in 1918, had not yet been noted. In the instances of England, France, the United States and some other countries, the epidemic influenza and cases of lethargic encephalitis were more or less coincidental. Since influenza varies so much in degree of severity, it is of little moment to debate whether or not victims of the encephalitis had previously suffered from influenza.

On the other hand, there is no recognized numerical relationship between the extent of influenza, and the number of cases arising, or at least identified, of the encephalitis. It is, of course, true that encephalitis has long been recognized as one of the sequels of epidemic influenza. Indeed, in the etiology of encephalitis, influenza occupies a prominent place; but in no other pandemic of influenza has this remarkable association of encephalitis occurred with certainty. Little weight can be given the supposed coincidence of influenza and the "sleeping sickness" of 1712; and it is highly improbable that the semi-mysterious affection, "nona," which dates from 1890, should have taken its origin from the influenza epidemic in southeastern Europe at that period and the association not have been observed elsewhere in Europe or even in America at the same time as a concomitant of the influenza epidemic, which raged with great intensity in those coun-

tries. Moreover, the occasional cases of encephalitis definitely observed to follow attacks of influenza have presented a more hemorrhagic character, and sometimes have been attended by Pfeiffer bacilli in the nervous tissues and meninges, which is not the case in the lethargic disease we are now considering.

Finally, should the reported experimental transmission of the encephalitis to animals be confirmed, a further distinction from the influenzal variety will have been established. Therefore, the outbreak of lethargic encephalitis either antedated (Austria) the pandemic of influenza of 1918, or (in other countries) the two diseases more or less overlapped, that is, although probably quite by accident, they prevailed concurrently. It is desirable, for the time being at least, to regard them as independent diseases.

The history of lethargic encephalitis indicates its infectious and also its communicable nature, but thus far single rather than multiple cases have been observed in family and other intimate groups of persons. However, two cases in a family have very rarely been noted; and in one instance an institutional outbreak has been reported in which among twenty-one inmates of a girls' home twelve cases arose, with five deaths.<sup>8</sup> Whether more accurate means of diagnosis, through which the nonlocalizing or "abortive" and the frankly paralytic lethargic cases would be more certainly associated and thus lead to a general revision of present views regarding multiple cases, can only be surmised. Obviously, in the interest of knowledge as well as of the prevention of the disease, close attention to this point is desirable.

It is now a matter of great importance to determine the precise nature or etiology of lethargic encephalitis. Many unsuccessful attempts have been made to communicate the disease to monkeys and other animals through the inoculation of nervous tissues showing the particular lesions, in the manner so readily and successfully employed in monkeys for poliomyelitis. This circumstance alone would serve to distinguish this epidemic encephalitis from epidemic poliomyelitis. But in two or three instances, what are stated to be successful transmissions of the disease to animals have been reported.

8. Forty-Eighth Annual Report of the Local Government Board, 1918-1919, Medical Supplement, London, 1919, p. 76.

Von Wiesner<sup>9</sup> of Vienna inoculated a monkey subdurally with nervous tissue from a fatal case of von Economo's. This animal quickly became severely sick and died in about forty-eight hours. At necropsy a meningo-encephalitis was found, and from the lesions a diplostreptococcus was cultivated. While von Wiesner regarded this experiment as successful, further investigation has indicated that the infection with the bacteria was an accidental and secondary process, and the diplostreptococcus is not etiologically related to lethargic encephalitis.

Loewe, Hirshfeld and Strauss<sup>10</sup> inoculated rabbits and monkeys with filtered extracts of the nasopharynx of cases of the encephalitis and, also, with filtered nasopharyngeal washings, and have induced a meningo-encephalitis in those animals. Apparently they did not succeed in infecting those animals by inoculating the affected nerve tissues themselves. They also believe that they have cultivated a minute organism, resembling the globoid bodies of poliomyelitis, which they think may be the inciting microbic agent of the disease. Discrepancies exist between the positive results of these authors and the many failures of others with similar inoculations which only greater experience can clear up.

Finally, McIntosh,<sup>8</sup> of the London Hospital, announced that a monkey inoculated with the material from the fatal cases in the home for girls, already referred to, presented lethargic symptoms and tremors and died. The brain on examination is said to have shown lesions similar to those found in human cases of lethargic encephalitis.

#### CONCLUSION.

The foregoing account represents, in brief, the present state of our knowledge of the interesting and important disease—lethargic or epidemic encephalitis. Obviously, that knowledge is still very imperfect. It is still too soon to say whether or not we are now at the threshold of the clearing up, by way of animal experiment, of the etiology and mode of communication of this menacing disease, as was accomplished so recently, and also by animal experiment, in the case of

9. Von Wiesner, R.: Wien. klin. Wchnschr. 30: 933, 1917.

10. Loewe, Leo, Hirshfeld, Samuel, and Strauss, Israel: J. Infect. Dis. 25: 377 (Nov.) 1919.

poliomyelitis. It is to be sincerely hoped that we are. But at this moment, and while waiting for the ultimate and convincing experimental results, one need entertain no doubt of the infectious and communicable nature of lethargic encephalitis.

The belief that lethargic encephalitis is a communicable disease imposes certain responsibilities on the medical profession to limit its spread. The outstanding obligation is perhaps the close study of suspected cases in order to determine their real nature, meanwhile holding them under such conditions of isolation as is usual with this class of diseases. Then every effort should be made to determine the existence of, and to detect and control the ambulant or abortive cases, having in mind that they may be more significant than the frankly lethargic and paralytic ones from the public health point of view. Since the nasopharyngeal secretions have become suspected of harboring the inciting microbic agent, adequate measures of controlling the distribution of those secretions into the surroundings of patients should be carried out. It is self-evident that the physician should invite the cooperation of pathologist and bacteriologist in attacking the unsolved problems presented by this unusual disease. It is to be hoped, indeed, that the disease may not secure a permanent lodgment in the country; on a wider knowledge of its occurrence and a deeper understanding of its nature, which the studies of the immediate future may yield, much, therefore, may depend.



[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, April 1, 1920, Vol. xxxi,  
No. 4, pp. 475-498.]

## EXPERIMENTAL SYPHILIS IN THE RABBIT.

### I. PRIMARY INFECTION IN THE TESTICLE.

By WADE H. BROWN, M.D., AND LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 45 TO 52.

(Received for publication, January 29, 1920.)

The successful transmission of the virus of human syphilis to rabbits in 1906 (1) gave promise of an unusual opportunity for the investigation of problems relating to syphilitic infection by the use of experimental means, and during the years following, a great deal of time was devoted to the study of the animal infection with a view to adapting it to this purpose. Numerous methods of inoculation were devised and perfected and the resulting infections were studied in great detail, but the hope of obtaining an experimental infection by local inoculation which was closely analogous to the human disease was not fully realized. Isolated instances of generalized infection with the occurrence of lesions of various types have been reported from time to time, and while some investigators have obtained such evidence of generalization in as many as 50 per cent of the infected animals (2), these occurrences have been comparatively rare in the experience of most observers.

It should be remembered, however, that much of the work which has been reported was based largely upon the study of animals inoculated with virus recently isolated from human sources or with virus which had been carried in rabbits for only a few years, and that while most investigators are of the opinion that the virulence of *Treponema pallidum* for rabbits may be increased by passage or by adaptation, it is still uncertain how long this increase in virulence can be kept up or to what extent it may be carried.

The infection in the rabbit, as in man, is one which runs a prolonged course and is very variable in character, so that it would

necessarily require years of observation and comparison of infections in large series of animals to reach any definite conclusion upon these points. Thus far, it is doubtful whether any one has been in a position to carry out such experiments upon a scale which would enable him to say what form of disease might ultimately be established in the experimental animal or how closely this disease might be made to resemble the human infection. From the standpoint of the use of the experimental infection as a means of investigating problems of human syphilis, it is obvious, however, that the animal infection should be brought to a stage of development as near that of the human infection as possible. From our own investigations it appears quite likely that this condition is much more nearly attainable than results thus far reported might indicate.

Among the problems of human syphilis which have been attacked through the use of the experimental infection in the rabbit, that of therapy stands out as of foremost importance, and our interest in experimental syphilis grew out of the use of the rabbit infection as a means of studying the therapeutic action of drugs. While at no time have we been able to undertake the study of the experimental disease as an independent problem, we have, nevertheless, been forced to carry out a great deal of collateral investigation as the need for information of a given character arose. In addition, we have had the opportunity of studying a large series of animals infected with two strains of *Treponema pallidum* both of which have been carried in rabbits for a number of years.

What we have to report at present, therefore, is not the result of a series of experiments designed expressly for the solution of particular problems in experimental syphilis, but the results of a series of observations supplemented by experiments intended to give information of a particular character.

The chief needs for therapeutic work upon experimental syphilis are reliable means of propagating the infection and an accurate knowledge of the disease as it exists in the experimental animal. It is the purpose of these papers, therefore, to condense the results of 4 years observation into a series of brief reports dealing with clinical aspects of experimental syphilis in the rabbit with especial reference to the phases of the subject which are of importance in carrying out

therapeutic experiments. Detailed discussion of individual papers will be deferred until the series has been completed, when the various aspects of the subject will be considered together. Later, we hope to be able to take up other phases of experimental syphilis which cannot be included in the present group of papers.

It will be found that our description of the experimental infection in the rabbit differs materially from others in the literature, but this is not to be taken as evidence of conflicting observations. We have dealt with older strains of *Treponema pallidum* than have commonly been used, and different principles have been employed in the handling of these organisms, so that it is not unlikely that the infection produced by us has been of a different order. Whether or not one uses such organisms to begin with, it is well to have in mind the state of virulence to which an organism may be developed and the character of the infection which may be produced by these organisms.

#### *Primary Infections in the Rabbit.*

Three general forms of local inoculation have been successfully employed in the rabbit. These include inoculation into the anterior chamber of the eye, inoculation into the testicle, and inoculation of skin surfaces—usually the scrotum. Of these, the first is at present chiefly of historical and scientific interest. Testicular inoculation was introduced by Parodi (3) in 1907 and soon became the most widely used form of inoculation. It is generally recognized that testicular infections are especially well adapted to the maintenance of pure strains of the organisms, but they are on the whole of less value as objects of clinical study, since a large part of the process takes place in parts hidden from direct observation, and the infection is of a less stable character than that produced by skin inoculations. There are, however, certain features of the syphilitic reaction which are brought out to greatest advantage by this form of infection, so that the consideration of the experimental disease may logically begin at this point.

## EXPERIMENTAL.

*Organisms Used.*—Two strains of *Treponema pallidum* were used in the work to be reported. One was obtained from Dr. Hans Zinsser and Dr. J. G. Hopkins and was isolated by them from a mucous patch on November 17, 1913, being Strain A of their series (4). The other organism was the Nichols nervous strain V for which we are indebted to Colonel M. A. Reasoner, Medical Corps, U. S. Army (2).

The first of these organisms has been carried in rabbits in our laboratory for 4 years and had been isolated 2 years before it came to us. The Nichols strain was isolated from the spinal fluid of a case of neurorecidive in June, 1912, and has, therefore, been carried in rabbits for more than 7 years.

*Animals Used.*—During the course of the work, many types of rabbits were used. There were many animals whose markings indicated either a pure stock or a strong admixture of such breeds as the Belgian hare, the Flemish giant, the Dutch belt, the Himalayan, the silver-gray, the Polish, the New Zealand red, the Angora, the English, and perhaps others. The majority of the animals, however, were of the common varieties of albino, gray, brown, brindle, black, blue or maltese, and animals of mixed or broken coloring. We have used animals of all ages and degrees of testicular development, from the young rabbit of 3 or 4 months in which the testicles were still but slightly developed to the very old rabbit with redundant scrotum and atrophic testicles.

From records showing the character of each rabbit used, its weight, testicular development, character of the scrotum, etc., it was found that in as far as the testicular infection alone was concerned, almost any type of animal with well developed testicles would give good results, but that if other features of the infection were to be considered also, the best results were obtained from the use of the small or medium sized albino and common gray or brown rabbits. As far as possible, animals were selected in which the testicles were well developed, with a preference for young rather than old animals.

*Technique of Inoculations.*—Testicular inoculations were carried out by the use of a virus emulsion prepared from an infected testicle or skin nodule. The animal which served as a source for the virus was anesthetized and the testicle or nodule excised with aseptic precautions. This material, or a portion of it, was then placed in a sterile mortar and finely minced with scissors, after which enough sterile salt solution (0.85 per cent) was added to moisten the entire mass. The material was then rubbed to a thick paste, more salt solution being added and thoroughly mixed with the contents of the mortar; the amount of salt solution to be added was determined by the dilution of the spirochetes in the emulsion as indicated by dark-field examination. The emulsion ordinarily used contained not more than two to three spirochetes to a microscopic field nor fewer than a single organism to two to three fields. Such an emulsion was usually obtained with from five to ten volumes of salt solution to one of tissue. The only object in attempting to control the strength of the emulsion was to insure

the presence of a sufficient number of organisms to produce a prompt reaction and to maintain some degree of uniformity in the dose of virus used in successive transfers.

The fluid emulsion was aspirated into a small glass syringe fitted with a 22 gauge needle  $\frac{1}{8}$  inch in length. The scrotum of the animal to be inoculated was sponged with 50 per cent alcohol, more for cleanliness than for antiseptic purposes, and 0.2 to 0.5 cc. of the emulsion was injected into the center of each testicle. The exact dose used was determined by both the strength of the emulsion and the size of the testicle to be inoculated. The precaution was taken to see that the needles used were sharp and smooth to avoid laceration and that the testicle was not unduly distended by the fluid injected.

This technique is one intended for intensive inoculations and never failed to produce a take. From time to time the procedure described was modified in several respects for different purposes, but we found it very simple of execution and entirely satisfactory for routine inoculations.

*Material Studied.*—From November, 1915, to September, 1919, 83 transfers of the two strains of *Treponema pallidum* were made by testicular inoculation. All the inoculated animals became infected and developed characteristic testicular lesions. Some were used for subinoculations and others for therapeutic experiments, but the course of the local infection was followed in a large number of animals from the time of inoculation to the spontaneous disappearance of the local lesions, and some animals were kept under constant observation for a much longer period of time.

#### *Local Reaction to the Infection.*

The infections produced in the testicles of rabbits inoculated with *Treponema pallidum* differed in many important respects. There were differences in the time and mode of onset and in the character and progress of the reaction as well as in the nature of the lesions developed, but on the whole the course of these infections tended to conform to a given plan and the variations were largely those of detail. The characteristic feature of the reaction observed in this series of animals was a tendency to the occurrence of a succession of changes separable into periods or phases of progression and quiescence or regression which gave to the local reaction a cyclic or relapsing course not unlike that which characterizes the infections produced by the blood spirochetes. This type of reaction occurred not only in the testicles but was the fundamental pattern of every phase of the syphilitic reaction in the rabbit, and forms, therefore, a logical basis for a consideration of syphilitic infections.

*Incubation Period.*

The time elapsing between inoculation and the development of testicular changes which could be recognized by external examination varied from 2 to 6 weeks, with occasional instances of longer or shorter periods of incubation. In fully 90 per cent of the animals, however, the incubation period fell within the narrower limits of from 3 to 4 weeks, and under properly chosen conditions of virus and rate of transfer, it was found that this time could always be brought within the 3 weeks period.

By dark-field examination of fluid obtained from the testicles, spirochetes were frequently demonstrated at a period well in advance of the appearance of clinical signs of infection. The same was found to be true of histological changes and not infrequently even gross alterations could be made out in the excised organ before any clinical sign of infection had become apparent. The time at which spirochetes and histological changes became demonstrable need not be reported in detail but was found to be within 7 to 10 days after inoculation.

Successive transfers did not necessarily shorten the incubation period to any appreciable extent. The dose of virus used, other things being equal, had a noticeable effect upon the speed of reaction, but this was not always the case. The vitality, or what may be termed the infectivity of the spirochetes, proved to be a factor of much greater importance than that of dosage. Thus, transfers made from actively developing lesions or from animal to animal as rapidly as the infection developed tended to produce or maintain a short incubation period, while inoculations made from old, inactive, or regressing lesions showed a relative prolongation of the incubation period irrespective of the dose of spirochetes used.

*Mode of Onset.*

The specific reaction in the testicle began in one of two ways, either as a small circumscribed focus of induration situated at the point of inoculation or as a diffuse swelling with increased tension of the entire testicle. When inoculations were made from old or inactive lesions or at relatively long intervals, the first form of reaction usually prevailed, but when transfers were made from one animal to

another in rapid succession, the reaction was more often of the second type. These differences in the character of the initial reaction were only temporary, as a rule, and tended towards a common type as the infection advanced.

#### *Course of the Reaction.*

Once the presence of a specific reaction became established, the progress of the infection was marked by certain changes such as enlargement and induration of the testicles which were common features of the reaction in all animals. The details of these changes necessarily varied in individual animals or even in the two testicles of the same animal, so that in general two extreme types of reaction with numerous modifications and variations became recognizable, depending upon the speed and sharpness with which successive changes occurred and the extent to which the several processes participated in the reaction.

In its highest form, the specific reaction in the testicle was characterized by a short incubation period, a diffuse onset and rapid development followed by a sharp crisis during which there was marked regression of the testicular lesions, a period of quiescence or inactivity, and one or more secondary cycles of active proliferation and quiescence or regression. Naturally, all these phases of the testicular reactions were rarely observed in their ideal form in one animal, but the several features of the reaction and some of its more common modifications may be illustrated by concrete examples.

*Acute Exudative or Fulminating Reaction.*—The first form of reaction to be cited is one which was characterized by an intense cycle of acute reaction terminating in crisis and followed by a slight secondary cycle of proliferative reaction. The case of orchitis shown in Figs. 1 to 6 furnishes an example of a reaction of this kind.

Fig. 1 shows the testicles at the time of inoculation. 15 days after inoculation, there was an increased tension and slight swelling of the testicles which progressed rapidly for about 6 days, the left testicle being more affected than the right (Figs. 1 to 3). At first, the scrotum was drawn tightly about the testicle, its vessels were slightly prominent, and there was a faint reddening of the skin surface (Fig. 2). On the 6th day, the reaction reached its height (Fig. 3), having

culminated in a diffuse congestion and marked edema of the scrotum most of which had developed within the last 24 hours.<sup>1</sup>

On the 7th day after the appearance of the specific reaction, there was a sharp change during which the congestion and edema of the scrotum subsided very rapidly and were followed somewhat more slowly by a decrease in the swelling of the testicle and a softening of the induration. Within 3 days, regression had reached the point indicated in Fig. 4. At the end of a week, the testicles had returned to approximately normal size and appeared as small, rather soft, atrophic masses with an area of thickening at the lower pole of the right testicle.

No further change was noted during the succeeding 10 days. Then on the 12th day, the mass at the lower end of the right testicle was found to be definitely enlarged and indurated. This marked the beginning of a second cycle of progressive reaction which at this time was limited to a circumscribed area in one testicle. 7 days later, the nodule in the right testicle had increased to nearly 1 cm. in diameter and a few small shotty nodules were palpable in the left testicle (Fig. 5).

The lesion on the right continued to develop for another week at which time it presented the appearance shown in Fig. 6. Meantime, the nodules on the left had disappeared completely and there was no further reaction in this testicle. During the next 2 weeks, the nodule on the right showed several short periods of quiescence and slight activity and then underwent complete resolution.

The difference in the intensity and extent of the reaction in the two testicles of this animal is of especial importance. It will be seen by comparing the photographs that the acute reaction in the left testicle was much more intense than that in the right, and that when the crisis came, regression in this testicle took place more rapidly and was more complete. In like manner, the secondary or proliferative reaction, while slight in both testicles, was decidedly less in the left than in the right. This relation between the intensity of the first cycle of reaction on the one hand, and the secondary reaction on the other was so constant as almost to establish a rule of inverse proportions between the first and second cycles of reaction in a given animal.

<sup>1</sup> At this stage of the infection, the scrotum and scrotal sac were filled with a gelatinous exudate or with a clear straw-colored or slightly blood-stained fluid which coagulated quickly on standing. The testicles also contained a fluid exudate of similar character which dripped freely when the organ was cut. Microscopic examination of these structures showed an inflammatory exudate composed chiefly of serum and fibrin with some polymorphonuclear leucocytes and red blood cells.

*Chronic or Proliferative Reaction.*—The second general type of reaction which occurred in the testicles of infected rabbits was one in which infiltration and acute exudative phenomena were subordinated to processes of proliferation. Reactions of this type showed, therefore, a more gradual onset and development of lesions with less marked cyclic alterations. In this group, the infection frequently began as a circumscribed focus of induration which gradually spread until the entire testicle became involved. Enlargement of the testicle took place more slowly as a rule than in the preceding group of cases and was associated with induration rather than increased tension of the testicle. The infection usually progressed steadily for upwards of 2 to 3 weeks by which time the testicle presented the appearance of a large, smooth or slightly nodular organ of extreme hardness. Congestion of the scrotum was comparatively slight, and edema, when present, was less marked than in the typically acute cases.

Instead of a pronounced crisis, progress of the reaction ceased more gradually and was followed by a period of inaction or of comparatively slow regression during which the testicles diminished in size and softened to a greater or less extent. If edema was present, it disappeared more rapidly, but at the end of the period of regression, the testicle still remained definitely enlarged and indurated. This residual induration was sometimes diffuse or uniform in distribution, while at others it was limited to certain areas or portions of the testicle.

Growth of the lesions was then resumed and continued with occasional remissions so that the progressive tendency of the reaction remained uppermost for a considerable period of time. The reaction in the testicles then subsided and the lesions resolved in much the same way as they had developed.

Two examples of this type of reaction are given in Figs. 7 to 9 and 10 to 12 which represent stages in the progress of the reaction in two animals of the same series. The incubation period in these two animals was 29 and 22 days respectively. In the first animal (Figs. 7 to 9), there were diffuse enlargement and induration which were first noted about 4 weeks after inoculation; the infection involved the scrotum as well as the testicles proper. The reaction reached its height in the left testicle 36 days after inoculation (Fig. 7) and in the right 39

days (Fig. 8). Both testicles then showed a slight regression (indicated in the photographs by a slight decrease in size) followed by renewed activity toward the end of the 7th week after inoculation (Fig. 9).

The reaction exhibited by the second animal differed somewhat from that of the first. There were again a diffuse enlargement and induration of both testicles, but, as indicated by a shorter incubation period, the reaction took place more rapidly, reaching its height during the middle of the 4th week after inoculation (Fig. 10). At the time the reaction was nearing its height in the first animal, a decided regression had already taken place in the second (Fig. 11). This regression was more marked than in the first case, and a well marked second cycle of reaction was not apparent until towards the end of the 7th week. The character of this reaction is indicated in Fig. 12 which shows the condition present 8 weeks after inoculation.

*Variations in the Specific Reaction.  
Subacute Reactions.*

The modifications and combinations of these two fundamental types of reaction were, as we have said, quite numerous, but a few specific examples will serve to indicate the character and direction of the more important variations.

The first variation which may be mentioned is one concerning the acute type of reaction. The photographs reproduced in Figs. 13 to 15 illustrate a reaction which was characterized by an acute cycle of moderate extent associated with some diffuse induration in the testicle which persisted after the crisis. The reaction began in this animal as a circumscribed focus of induration in the posterior portion of the testicle, which was first detected 18 days after inoculation. Induration spread and the testicle enlarged rather rapidly, at the same time becoming diffusely indurated. The height of this change was reached 26 days after inoculation (Fig. 13). There was a moderate edema of the scrotum which lasted for a few days and then subsided, leaving the testicle still slightly enlarged as indicated in Fig. 14. There was some further regression of the lesions, and renewed activity did not set in until towards the end of the 7th week. This second cycle of reaction was both focal and diffuse in character and at the time the third photograph of the series was taken (Fig. 15) there were diffuse induration of both testicles and numerous nodules varying from a few mm. to approximately 0.5 cm. in diameter.

A second type of orchitis which was quite common in our series of animals is shown in Figs. 16 to 18. The onset of the infection in this instance was again of a circumscribed nodular character with an incubation period of 27 days. The testicles became diffusely indurated and showed a marked enlargement. This reaction took place somewhat more slowly than in the preceding case and did not

reach its full development until about 6 weeks after inoculation (Fig. 16). At the time the first photograph was taken (Fig. 16) the left testicle was retracted within the abdominal cavity and could not be brought through the inguinal canal, and there was very marked edema of both scrotal sacs which is best shown on the right. Edema subsided rather slowly, and at the same time the testicles diminished somewhat in size and became slightly softened. On the 49th day (Fig. 17) the scrotum showed a diffuse thickening with beginning induration over the ventral surface of both testicles; the testicles themselves were still considerably enlarged and markedly indurated. From this point onward, the reaction gradually shifted from the testicles proper to the scrotum with the production of chancre-like lesions, the beginning of which is indicated in Fig. 18.

The feature of especial interest in the reaction exhibited by this animal was the slowly progressive but pronounced character of the reaction, culminating in a diffuse swelling and edema of both the testicles and the scrotum and the subsequent transference of the center of reaction from the testicles to the scrotum—the latter condition being a frequent occurrence in testicular infections.

Another group of photographs illustrating an infection of somewhat the same character is reproduced in Figs. 19 to 22. This animal was an old albino with rather atrophic testicles and, as is usually the case with such animals, the reaction was slow to develop. Infection began as a circumscribed focus of induration which was recognized about 4 weeks after inoculation and gradually spread until the entire testicle was diffusely involved. There was a slow but steady increase in the size and induration of the testicles, extending over a period of about 2 weeks. The height of the reaction in the right testicle was reached 46 days after inoculation (Fig. 19) and a few days later in the left. There were moderate congestion and edema of the scrotum which lasted for several days. Crisis occurred, followed by regression, but at the end of this phase of the reaction, both testicles were still diffusely enlarged and indurated much as they appear in Fig. 20. Within 10 days after the crisis, there was renewed growth of lesions situated in the tail of the epididymis (Fig. 21). These lesions grew actively for another 2 weeks at which time they formed large indurated masses of irregular shape, involving the skin as well as the epididymis (Fig. 22). In the meantime, the reaction in the remaining portions of the testicle had completely subsided, leaving the testicles as small atrophic masses.

A fourth example of a more unusual reaction is given in Figs. 23 to 25. The earlier stages of the reaction in this animal were of an ordinary chronic proliferative type and had progressed to the point of the formation of a scrotal chancre on the left associated with induration of the testicle itself with marked enlargement and induration of the right testicle and slight extension to the scrotum at its lower pole, when, towards the end of the 7th week, there was an acute exudative reaction in the right testicle and scrotum, which reached its height on the 53rd day after inoculation. This reaction was followed by a typical crisis (Figs. 23 and 24), and both testicles then began to diminish in size and induration,

while the lesions of the scrotum continued to grow actively (Fig. 25). The feature of especial interest here was the occurrence of an acute exudative reaction late in the course of infection.

This small group of cases, including the more acute and chronic forms of reaction together with various modifications and combinations of these two fundamental types of processes, is typical of the reactions seen in practically all cases of outspoken testicular infection. Many of these modified reactions might be spoken of as subacute in the same sense in which the others are acute or chronic in character, since they combine to a greater or less degree the features of exudation and infiltration with those of proliferation of fixed tissue cells. It will be noted, however, that whatever the variation in the response of the individual animal, they all show an unmistakable tendency towards a reaction of a recurrent or a relapsing type.

It was found that these reactions could be influenced to a considerable extent in several ways. Thus, reactions of the more acute type were especially frequent when transfers were made from one animal to another in rapid succession or during the ascending phase of the acute reaction. In a small proportion of animals, the local infection terminated with a single cycle of reaction such as that described. As a rule, however, the acute cycle of reaction was followed by other changes leading to the formation of lesions differing in many ways from those originally produced. The occurrence of these secondary cycles of reaction depended to a considerable extent upon the character and extent of the first cycle. Thus animals in which there was an intense, acute reaction were, as a class, less apt to show secondary reactions of marked degree than those in which the process had been more gradual or less intense.

Reactions tending towards the chronic or proliferative type were quite common and occurred with especial frequency after inoculations made at long intervals or from chronic indurative lesions. They were frequently associated with the most persistent local infections and gave rise to some of the most conspicuous and destructive lesions of the testicles.

*Late Developments of the Testicular Reaction and the Character of the Lesions Produced.*

Most writers have treated the testicular infection of the rabbit from the standpoint of the lesions produced, but we prefer to regard these lesions more as manifestations of a reaction to infection and hence very little has been said concerning the lesions themselves. Following out their ideas of the importance of the lesion as an entity in itself, many investigators have attempted to differentiate between the various types of lesions produced, and several classifications have been proposed, based largely upon the location of the lesion and the character of the pathological process (5, 6). In our experience such differentiations would be very difficult to make except as applied to a particular lesion at a particular time or to what may be termed the residual lesions which ultimately come to be established.

Without entering into a detailed discussion of this phase of the subject, it may be said that while the infection in the animals studied by us began either as a circumscribed or as a diffuse process, neither of these conditions was permanent, and the same applied to the character of the pathological process. In practically all instances, the infection ultimately involved the entire organ with the production of lesions in the parenchyma, tunics, epididymis, and cord, and in many instances in the scrotum as well. This extension took place early, as a rule; and by the time the reaction had reached its height, there was what might be termed a panorchitis.

In many animals, this widespread involvement was of a perfectly uniform character as far as could be determined by the gross appearance of the organ or by palpation. In a second group of animals, there was a finely granular condition of the testicle, while in a third, distinct indurated nodules could be recognized, separated by tissue showing a lesser degree of involvement or an involvement of a different character. This differentiation into lesions of a particular type or lesions situated in certain localities came as a late development in the course of the infection and was, in our opinion, attributable to the character of the reaction against the local infection and the tendency on the part of the infection to extend in certain directions. During the later stages of the infection, therefore, one fre-

quently had to deal not with a widespread infection at the height of its activity but with an infection which was localized only in certain areas. The points of most frequent involvement were the globus minor and major, the tunics, the mediastinum testis, and the skin, or, in other words, the areas in which membranes or connective tissue in some form was most abundant.

These later developments and extensions of the testicular infections are of considerable interest both from the standpoint of the reaction to infection and the lesions which are produced. Several examples of this phase of the local reaction have already been given in the preceding illustrations, which may be supplemented by Figs. 26 to 37.

The first group of photographs in this series (Figs. 26 to 28) illustrates an infection which began as a diffuse indurative orchitis. At the period of infection represented in Fig. 26, 58 days after inoculation, a more or less diffuse extension of the infection to the scrotum of the right testicle had taken place, while on the left, the lesions in the testicle and scrotum were assuming a more nodular character, and these differences in the character of the changes in the two testicles persisted to the end. As the infection advanced, a large portion of the right testicle and scrotum underwent diffuse necrosis; on the left, the lesions began to be more circumscribed and two well defined scrotal lesions were formed (Fig. 27). Eventually, a large portion of both testicles underwent necrosis as indicated in Fig. 28, which shows the condition 80 days after inoculation. Even at this stage, however, the multinodular character of the lesions of the left testicle could still be made out, there being three fairly well defined centers of reaction.

In this animal, the feature of especial importance in the reaction was the tendency to widespread necrosis which eventually involved the entire skin surface and the testicle as well. This was not always the case, however, even when the reaction in the testicle was fully as marked as in this instance. In Fig. 29, a case of orchitis is illustrated in which the involvement of the testicle was also quite marked, but the necrosis and ulceration were confined to two more or less circumscribed areas producing effects more analogous to primary skin lesions. Lesions of this type were very common among our animals, and all gradations and transitions could be found between the lesion which was definitely a diffuse orchitis with necrosis and ulceration and lesions which were identical in character with primary skin lesions. The photographs reproduced in Figs. 30 to 32 are given to illustrate this point.

Fig. 30 shows a condition which is clearly an orchitis with skin involvement which has led to the formation of depressed ulcers with a definitely indurated collar such as is seen in primary skin lesions. In Fig. 31, taken only 6 days later, the picture has changed somewhat, and the whole mass in the left testicle

has filled out, while the right testicle now shows a single large ulcerated lesion identical with the large skin chancres which develop in the rabbit.

The next step in the series of transition is shown in Fig. 32. The infection in this animal began as a diffuse indurative orchitis which later became nodular with lesions such as those shown in Fig. 21, except that there was an active nodule situated in the tunic of the left testicle. The nodule in the epididymis of the right testicle extended to the skin, forming a chancre-like mass seen in Fig. 32. On the left, the nodule in the tunic was the one to develop most actively, forming a skin lesion still more like the ordinary chancre. These lesions healed in the course of about 4 weeks, leaving an active nodule in the epididymis of the left testicle (Figs. 33 and 34).

A final transition in the course of the testicular infection may be illustrated by Figs. 35 to 37. The infection in this animal began as a circumscribed focus of induration which quickly developed into a diffuse orchitis (Fig. 35). In time, the diffuse induration resolved, leaving small focal lesions in the tail of the epididymis of both testicles. Foci of infection then appeared in the skin and developed into the lesions shown in Fig. 36 (77 days after inoculation). Upon removal of the testicles and skin lesions, the testicles were found to be small atrophic masses entirely free from any gross evidences of active infection (Fig. 37). On the right, there was an indurated lesion in the epididymis which in part was continuous with the skin lesion; on the left, the two processes showed less connection and one of the two lesions in the scrotum was entirely distinct from the other lesions present (Figs. 36 and 37).

In this animal an infection which began as a circumscribed focus of induration first became diffuse, was then transformed into a nodular epididymitis, and the seat of active infection was finally transferred to the scrotum, leaving the body of the testicle free from active lesions.

#### *Atypical and Low Grade Reactions.*

Before leaving the subject of the local reaction in the testicle, mention should be made of another class of infections. There were, in our series, a few animals which showed some peculiarity in their response to infection, such as the development of slight or otherwise atypical lesions. These infections may be spoken of as atypical, on account of such peculiarities in the reaction, or as low grade reactions, in the sense that the reaction lagged or that the lesions which developed were of a minor character.

The common instances of this kind were slight reactions of short duration and slight but persistent reactions. Of the latter class, there were two main groups of infections, one in which a diffuse or

circumscribed focus of reaction developed very slowly and never resulted in more than a slight diffuse thickening in the testicle or a small nodular induration. The second group included instances in which a slight diffuse reaction subsided with the formation of residual nodules or in which an initial focus of infection was later supplemented by the appearance of secondary foci of a similar character. Minor lesions such as these not infrequently persisted for months without showing any especial sign of activity or growth.

Most of the infections of this class occurred during the earlier part of the work and have rarely been seen during the last 2 years. We are inclined to attribute occurrences of this kind to the circumstances under which the transfers were made.

From our present knowledge of conditions which influence the specific infection in the rabbit, we recognize this class of cases as attributable to certain definite causes, mainly to an attempt to inoculate with spirochetes at a time when their vitality, or infectivity, had been materially reduced by cyclic immunological reactions (page 492), but also to peculiarities in the response of individual animals to the specific infection.

#### *Accessory Skin Lesions.*

For the sake of completeness, mention may be made also of the occurrence of accessory lesions in the scrotum which develop along the path of the needle as a direct result of the process of inoculation (Figs. 2 and 4). At times, these lesions appeared before any reaction in the testicle had become recognizable. Without going into a discussion of this feature of testicular infections, which properly belongs in the section on scrotal chancres, it may be said that these foci of infection appeared in one of three forms, as small gelatinous swellings in the skin, as translucent pearly nodules in the superficial layers of the skin, or as opaque points or plaques in the depths of the scrotum, usually upon the outer surface of the tunica vaginalis. They rarely developed to lesions of any considerable extent. In some instances, however, they did develop into large lesions either as independent foci of reaction or as indistinguishable parts of the testicular reaction.

*The Spirochete Reaction and the Spirochete Content of Lesions.*

The above observations upon the course of the specific reaction in the testicle were supplemented by parallel dark-field examinations of fluid taken from the testicular lesions at different periods of the infection in order to determine what changes, if any, could be demonstrated among the infecting organisms while the changes in the testicles were in progress. From these examinations, it was found that as the infection progressed, the spirochetes exhibited changes analogous in character to those described in the testicles themselves. As we have already mentioned, spirochetes began to multiply and were present in considerable numbers before any gross manifestation of infection had become recognizable. This increase in spirochetes continued parallel with the development of the lesions, so that by the time the lesions had reached the height of their first cycle of development, actively motile spirochetes were present in large numbers.

At this point, the spirochetes suddenly began to lose their motility and to collect in tangled masses. In cases of acute orchitis, these changes coincided roughly with the development of edema of the scrotum and were followed promptly by regression of the testicular lesions.

Following the phenomenon of agglomeration, the spirochetes rapidly diminished in numbers, so that within a few days, organisms were difficult to find in fluid aspirated from the testicles, and those seen were either degenerated or showed but slight signs of motility; in many instances no organisms could be found.

After passing through a crisis such as this, actively motile spirochetes again appeared in the testicular fluid, and increased in numbers, presaging a renewed activity on the part of the lesions or at least a cessation of regression for the time being. These parallel changes continued throughout the existence of the local infection—the change in spirochetes usually occurring slightly in advance of the changes in the lesions.

As in the case of the lesions, cyclic changes were at times very sharp and easily recognizable, while at others they were less marked and might readily have escaped detection had it not been for the example of the sharper type of reaction.

During the latter part of the infection, the spirochetal content of lesions was found to be more difficult of estimation and somewhat uncertain. Where a single lesion was present, diffuse or circumscribed, the spirochetes in one area might diminish or disappear while they were present in considerable numbers or were actively increasing in other areas. These changes in the spirochetal content had their parallel, however, in the shifting centers of growth in the lesions themselves or, in the case of multiple lesions, in the resolution of one lesion while another was undergoing active development. By careful study of the lesions, it was found, however, that actively growing lesions or portions of lesions always contained actively motile spirochetes, and the same was true of many lesions which were merely quiescent but not regressing. The spirochetal content of lesions which were regressing was more variable; in many instances, spirochetes could not be detected by dark-field examination, while in others, they could still be demonstrated in fair numbers which diminished as the lesions resolved.

The changes affecting the spirochetes within the lesions will be recognized as entirely analogous to those which occur in blood stream infections with such organisms as *Spirocheta recurrentis*. In order to assure ourselves that the apparent immobilization, agglomeration, and degeneration described, entailed some actual alteration in the pathogenic properties of these organisms, tests were carried out by animal inoculation. For this purpose, inoculations were made with organisms taken as nearly as possible at the height of the first cycle in cases of intense, acute reaction, or more properly at the beginning of the crisis, and the tests were controlled by a parallel series of inoculations made with actively motile spirochetes taken from an early stage of testicular infection.

From these tests it was found that the infecting power of such organisms was markedly diminished. In one experiment, a series of animals inoculated with approximately ten times the dose of immobilized and agglomerated organisms that was used in the controls showed an incubation period of 6 weeks as contrasted with 3 weeks in the controls, while the lesions were slow to develop and were less pronounced than in the control animals. Similar observations as to the infectivity of the spirochetes at different periods of the infection have

been made many times and there is no doubt in our minds as to the significance of the cyclic reactions described.\*

#### *Duration of the Local Infection.*

The duration of the local infection as determined by the presence of active lesions was as variable as the course of the infection itself, and no fixed limits can be given either for the several phases of the local reaction or for the infection as a whole. The period of active infection varied anywhere from 1 to more than 12 months. In some animals, the entire reaction was represented by one intense cycle of acute reaction which terminated within 4 to 6 weeks after inoculation; in others, the infection continued through successive cycles of reaction, but the period of active infection was rarely longer than 2 to 4 months. Inactive or latent lesions which showed occasional periods of slight activity frequently persisted much longer, and residual lesions in the epididymis, skin, and tunics not infrequently persisted for from 4 to 8 months. In general, the duration of the local infection was inversely proportional to the intensity of the local reaction.

#### CONCLUSIONS.

The conclusions which might be drawn from this series of observations are very numerous, but we shall refer briefly to only a few of the more important points.

From the standpoint of a pathological process, it is important to note that the local response is not altogether a granulomatous reaction. In fact, it appears that exudation and infiltration are the fundamental processes and that proliferation is a secondary phenomenon.

In the second place, it is quite clear that the reaction to infection in the testicle, and hence the course of the infection itself, are of a periodic or relapsing character, analogous in this respect to other spirochete infections, or for that matter present certain protozoan

\* We have evidence sufficient to indicate that rabbits inoculated in the testicles or scrotum with *Treponema pallidum* always show a blood stream invasion, and that these organisms are subject to changes analogous to those which occur in the testicles. The details of this work will be reported later.

characteristics. To what extent this infection is influenced by a local reaction and to what extent by a systemic reaction are at present unknown, but as far as the local infection is concerned, it appears to be subject more to local than to general conditions. Further than this, it appears that the character of the reaction which takes place in a given case is itself significant. The relation between the reaction in the individual animal and the duration of the infection represents a constant. When the local reaction assumes the character of a chronic proliferative process, the life of the infection is prolonged and the extent of the reaction which takes place before the infection is brought under control is proportionately increased; when, however, the reaction assumes the form of an intensely acute reaction, the life of the local infection is promptly terminated. This relation between the local reaction and the duration of an active infection, together with the determination of the character of the experimental infection in the rabbit, are perhaps the two most important deductions to be drawn from this series of observations in that they touch every phase of the experimental infection.

#### SUMMARY.

A study was made of the infections produced in rabbits inoculated in the testicles with two strains of *Treponema pallidum* which had been carried in rabbits for several years. Infection resulted in all instances; the incubation period varied as a rule between 2 and 6 weeks and under properly chosen conditions could be reduced to approximately 3 weeks or less.

The resulting infection pursued a typically cyclic or relapsing course which affected both the spirochetes and the associated lesions in the testicle. The spirochetes in the local lesions exhibited periodic changes less marked and less regular but identical in character with the changes which occur in the blood in cases of relapsing fever. The lesions in the testicle also showed periods of active development and quiescence or regression which followed closely upon the changes exhibited by the spirochetes.

The specific reaction in the testicle showed considerable variation in the speed and sharpness with which successive phenomena occurred

as well as in the character and extent of the processes themselves. These reactions were of two fundamental types. In one group of animals, the reaction was characterized by an intense cycle of acute exudation and infiltration with a lesser degree of proliferation, followed by crisis and subsequent recurrence of secondary cycles of proliferative reaction of a minor degree. In the other group of animals, the reaction was more chronic in character and consisted largely of infiltration and proliferation. The progress of the reaction was more gradual, and sharp alterations in its course were absent. The infection progressed by a succession of stages with slight and irregular remissions.

In a third group of animals, the reaction was subacute, combining at the same time the processes of exudation, infiltration, and proliferation. The first cycle of reaction was fairly acute and terminated in a definite crisis with moderate regression which in turn was followed by recurrence and more or less pronounced secondary cycles of proliferation.

In all cases of outspoken infection, there was diffuse involvement of testicle, tunic, epididymis, and cord, but as the infection progressed, the lesions underwent many transformations, so that a variety of lesions was formed from processes which in the beginning were of a common type. Eventually, the reaction became more irregular and the infection became centered in one or more foci which were commonly situated in the epididymis, tunics, scrotum, or mediastinum testis. These centers served as residual foci of infection.

The duration of the testicular process was found to be very variable. In some animals, the entire reaction consisted of but a single sharp cycle, and the local infection was terminated by crisis within 4 to 6 weeks after inoculation. As a rule, the period of active infection was from 2 to 4 months, and quiescent or inactive lesions not infrequently lasted for from 4 to 6 months. In exceptional instances, local infection persisted for more than a year.

## BIBLIOGRAPHY.

1. Bertarelli, E., *Centr. Bakteriol., Ite Abt., Orig.*, 1906, xli, 320; 1907, xlili, 238.
2. Nichols, H. J., *J. Exp. Med.*, 1914, xix, 362.
3. Parodi, U., *Centr. Bakteriol., Ite Abt., Orig.*, 1907, xliv, 428.
4. Zinsser, H., Hopkins, J. G., and McBurney, M., *J. Exp. Med.*, 1916, xxiii, 329.
5. Uhlenhuth, P., and Mulzer, P., *Arb. k. Gsndhtsamte.*, 1913, xliv, 307.
6. Reasoner, M. A., *J. Am. Med. Assn.*, 1916, lxvii, 1799.

## EXPLANATION OF PLATES.

The figures are reproductions of untouched photographs which represent the objects at their natural size. The statements of time refer in all instances to the time after inoculation, unless otherwise stated.

## PLATE 45.

FIGS. 1 to 6. An acute diffuse reaction in the testicles and scrotum with crisis and regression followed by a slight secondary cycle of reaction and the formation of circumscribed nodular lesions.

FIG. 1. The normal testicles.

FIG. 2. 20 days. Well advanced, acute orchitis with beginning congestion and edema of the scrotum.

FIG. 3. 24 hours later. The height of the first cycle of reaction with intense congestion and edema most marked in the left testicle.

FIG. 4. 72 hours after the crisis. Note the greater regression of the left testicle.

FIG. 5. 48 days. The second cycle of reaction and the formation of nodular lesions in both testicles.

FIG. 6. 56 days. The lesion in the tail of the epididymis and scrotum of the right testicle is still increasing, while the lesions have disappeared from the left.

## PLATE 46.

FIGS. 7 to 9. Chronic proliferative orchitis with scrotal involvement.

FIG. 7. 36 days. The height of the first cycle of reaction in the left testicle. Note the perfectly uniform enlargement of the testicle extending even to the cord. The scrotum of both testicles is involved, and on the right, there is an area of beginning necrosis.

FIG. 8. 39 days. The height of the first cycle of reaction in the right testicle; the crisis in the left has passed and the lesions are regressing.

FIG. 9. 51 days. The second cycle of reaction in progress in both testicles. Note the smooth, tense scrotum of the right testicle indicative of diffuse involvement, while in the left the lesions are now assuming a multinodular character.

FIGS. 10 to 12. Chronic proliferative orchitis in another animal of the same series as that in Figs. 7 to 9.

FIG. 10. 24 days. Well marked diffuse induration of both testicles approximately at the height of the first cycle of reaction.

FIG. 11. 36 days. Decided regression of the lesions of both testicles.

FIG. 12. 56 days. An early stage of the second cycle of reaction.

#### PLATE 47.

FIGS. 13 to 15. An acute diffuse reaction of moderate degree followed by crisis and a second cycle of chronic proliferation, diffuse and focal in character.

FIG. 13. 26 days. The height of the acute reaction with moderate edema of the scrotum. The left testicle is larger than the right.

FIG. 14. 28 days. Regression. The left testicle is still larger than the right due to a greater degree of proliferation in the initial reaction. Compare Figs. 13 and 14 with Figs. 3 and 4.

FIG. 15. 53 days. Moderate enlargement and induration of both testicles.

FIGS. 16 to 18. Subacute orchitis characterized by marked induration and edema of both testicles and scrotum and gradual shifting of the center of reaction from the testicles to the scrotum.

FIG. 16. 41 days. The left testicle is retracted within the abdominal cavity.

FIG. 17. 49 days. Some regression has taken place, but the scrotum is thickened and both testicles are still diffusely enlarged and indurated.

FIG. 18. 52 days. The lesions in the testicles are still slowly diminishing, but focal lesions are beginning to develop in the scrotum.

#### PLATE 48.

FIGS. 19 to 22. A subacute reaction with diffuse involvement of both testicles followed by a second cycle of reaction localized in the tail of the epididymis.

FIG. 19. 46 days. The height of the first cycle of reaction in the right testicle.

FIG. 20. 53 days. The extent of the regression following the crisis in the reaction is shown. Note that at the lower end of the right testicle, there is a nodule which has not regressed to the same extent as the rest of the testicle.

FIG. 21. 60 days. Continued regression of the testicular lesions with development of focal lesions in the epididymis.

FIG. 22. 76 days. Focal lesions of the epididymis and scrotum.

#### PLATE 49.

FIGS. 23 to 25. An acute exudative reaction occurring during a late stage of the local infection.

FIG. 23. 53 days. Chronic proliferative changes in both testicles with localized lesions of the scrotum with acute congestion and edema on the right.

FIG. 24. 2 days later. Regression on the right with slight swelling of the testicle and increasing growth of the scrotal lesion on the left.

FIG. 25. 61 days. Regression of the testicular lesions with simultaneous growth of the lesions in the scrotum of both testicles.

#### PLATE 50.

FIGS. 26 to 28. Chronic orchitis with extensive necrosis of testicles and scrotum. Right diffuse; left nodular.

FIG. 26. 58 days. Diffuse induration of the right testicle and scrotum with beginning skin necrosis and multinodular lesions of the left testicle.

FIG. 27. 73 days. Diffuse necrosis of the right testicle and scrotum; focal necrosis of the left.

FIG. 28. 80 days. Later stage of same lesions. Note lines of demarcation between the three masses on the left.

#### PLATE 51.

FIG. 29. Chronic diffuse orchitis with circumscribed lesions of the skin resulting from metastatic infection of the testicles.

FIG. 30. Chronic diffuse orchitis with multiple chancre-like lesions of the skin.

FIG. 31. Same testicles 6 days later showing transformation of the lesions. Note especially the single large chancre-like mass in the right testicle.

FIGS. 32 to 34. Late transformations in a case of testicular infection.

FIG. 32. Chancre-like extensions to the scrotum.

FIG. 33. Healing of the skin lesions with an active nodule persisting in the epididymis of the left testicle.

FIG. 34. 93 days later. Skin lesions practically healed, while the nodule in the epididymis is still active.

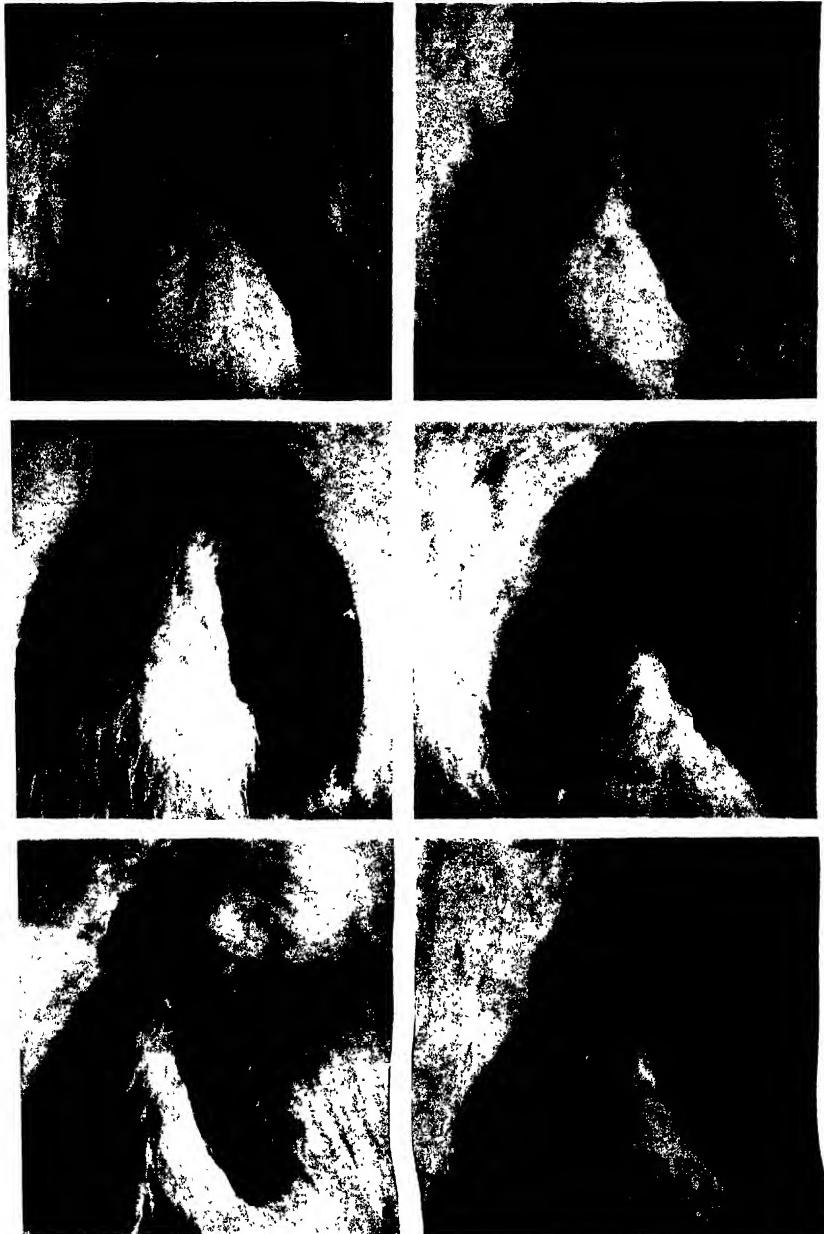
#### PLATE 52.

FIGS. 35 to 37. Diffuse orchitis with eventual transference of the local infection from the body of the testicles to the epididymis and scrotum.

FIG. 35. Diffuse indurative orchitis of both testicles.

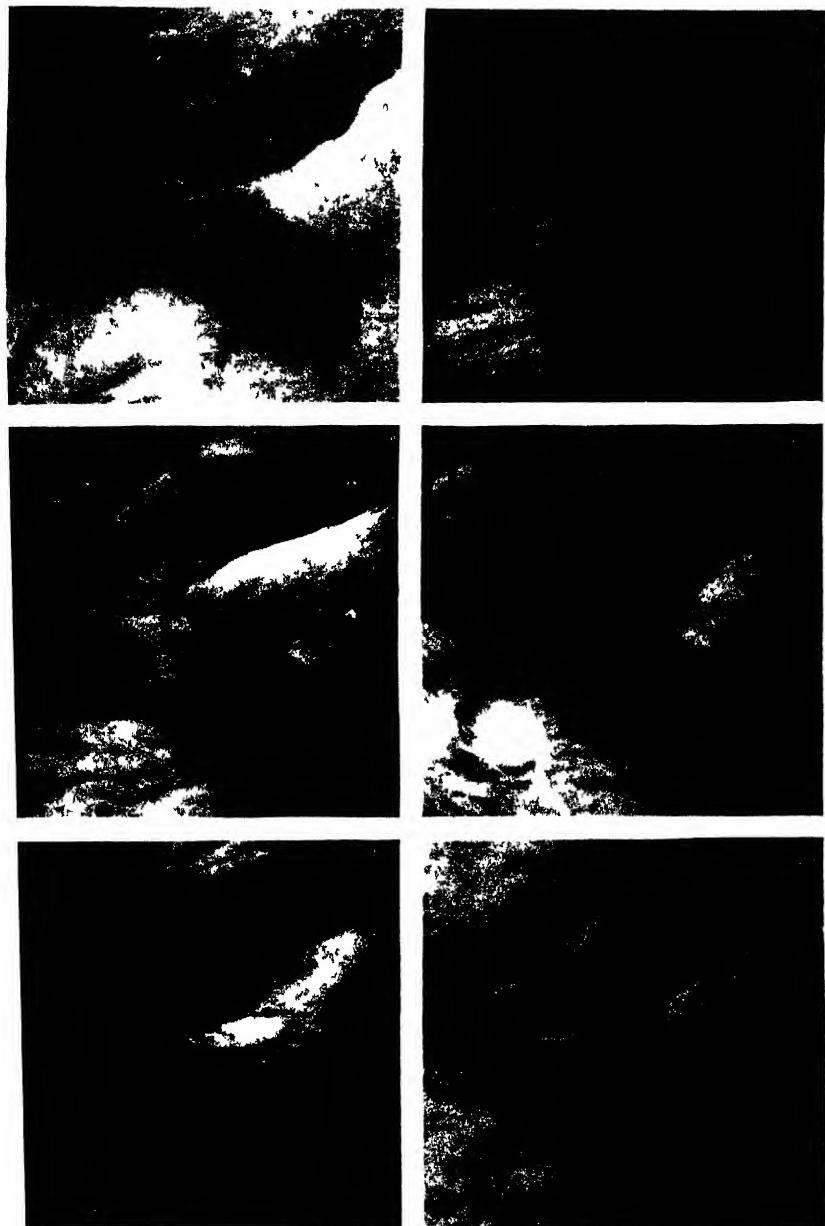
FIG. 36. The final lesions in the epididymis and scrotum.

FIG. 37. The same lesions excised and sectioned. Both testicles are atrophic; on the right, the nodule in the tail of the epididymis fits into a hollow beneath the ulcer in the skin, but the two lesions are not entirely fused with each other; on the left, there is only a small nodule in the epididymis which is fairly distinct from the two lesions in the scrotum.



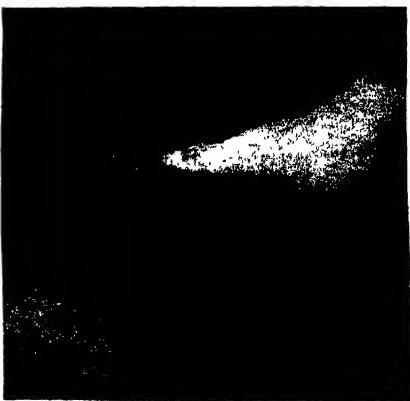
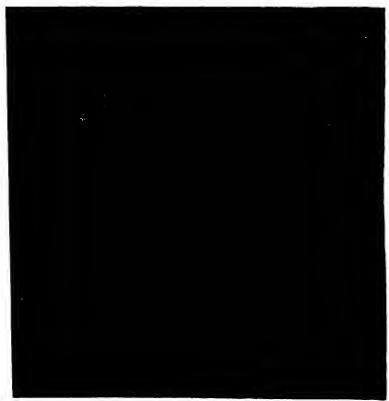
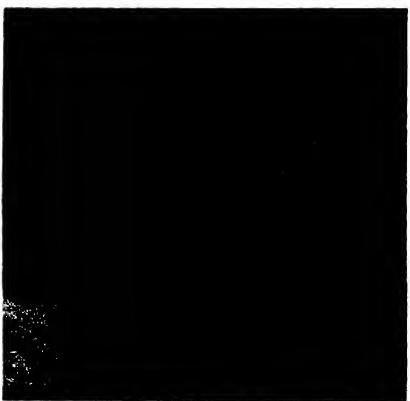
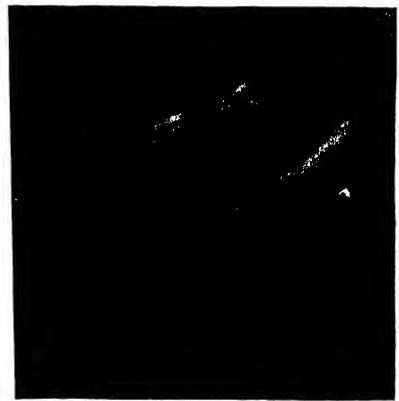
(Brown and Pearce: Experimental syphilis in the rabbit. I.)





(Brown and Pearce Experimental syphilis in the rabbit I)





(Brown and Pearce: Experimental syphilis in the rabbit. I.)





(Brown and Pearce—Experimental syphilis in the rabbit—D)





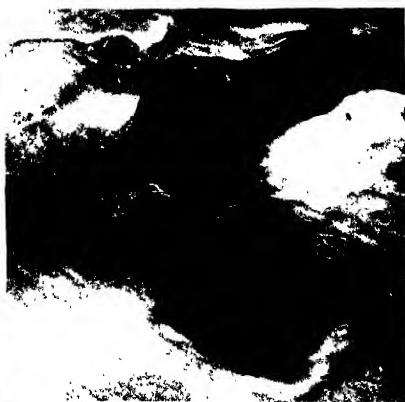
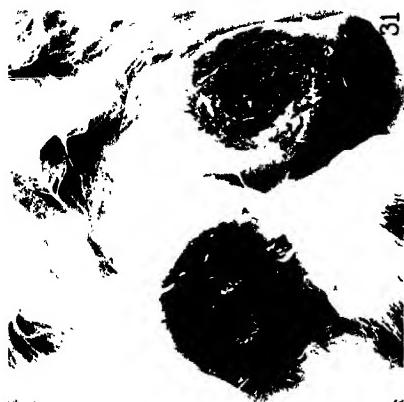
(Brown and Pearce—Experimental syphilis in the rabbit—1.)





(1 - week and 1 month Experimental syphilis in the rabbit 1)





(Brown & Pearce—Experimental syphilis in the rabbit—1)







## RELATION OF THE PORTAL BLOOD TO LIVER MAINTENANCE.

### A DEMONSTRATION OF LIVER ATROPHY CONDITIONAL ON COMPENSATION.

By PEYTON ROUS, M.D., AND LOUISE D. LARIMORE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 67 TO 71.

(Received for publication, February 2, 1920.)

In the course of observations on the rôle of the liver in blood formation and destruction, we have had occasion to ligate portal branches to the organ. The ensuing changes have been of such striking character as to merit study for their own sake; and the present paper is concerned with them. There already exists, of course, a considerable literature on so obvious a theme. For the moment it may suffice to state in this connection that according to the generally accepted view occlusion of a portal branch to the liver has no effect on the organ save when a grave derangement of the systemic circulation is also present. The complete local parenchymal atrophy that in our experiments regularly followed such occlusion was unforeseen, as was the further observation that the atrophy is conditional, being dependent upon a compensatory hypertrophy of the remainder of the organ.

#### *Method.*

The liver of the rabbit is singularly adapted for experiments involving the blood vessels and bile ducts, since it consists of two separate masses, each with its own vessels and ducts. The rabbit may indeed for operative purposes be said to possess two livers. They are of very unequal size, the larger, or main liver, as we shall call it, formed of the left anterior and posterior lobes and the right anterior lobe with the gall bladder, being three times as big as the smaller, or lobe mass, which consists of the right posterior and caudate lobes. The lobe mass contains just enough parenchyma, as Ponfick<sup>1</sup> showed, to suffice for the

---

<sup>1</sup> Ponfick, E., *Virchows Arch. path. Anat.*, 1889, cxviii, 209; 1890, cxix, 193.

needs of the organism when the main liver is ablated. Through it, under such circumstances, all of the portal stream finds room to pass.

In our experiments the portal trunk to the main liver of the rabbit has been ligated just above the caudate lobe. By such means the whole portal stream is diverted to the lobe mass. The caudate lobe, though a part of the latter by reason of its parenchymal connection with the right posterior lobe, has an added source of venous blood through a small branch arising from the portal trunk at the level of the ligature and frequently compromised by it. To avoid the irregularity thus introduced, the caudate lobe was tied off and cut away as a routine measure. A piece of the tissue was sectioned to determine the condition of the liver.

In ligating the portal trunk great care was taken not to interfere with the main bile duct and hepatic artery. The operation was carried out under ether on rabbits of from 1,400 to 2,300 gm. Closure was done in three layers. Occasionally a fatal necrotic process spread from the ligated caudate stump, but the great majority of the animals recovered without complication and remained in perfect health. They were killed with chloroform at periods of from 12 days to 6 months after the original operation. The liver masses were weighed separately after the blood, as yet unclotted, had flowed away from the severed vessels. This, it was felt, would result in a truer estimate of relative amounts of parenchyma than if the blood were retained by closing the vessels, as was Ponfick's method. According to Ponfick,<sup>1</sup> the normal liver of the rabbit averages 3.56 per cent of the gross body weight, though he prefers to use 4 per cent in calculations. On his estimation the main mass forms 74.7 per cent of the total, the right posterior lobe 19.3 per cent, and the caudate lobe 6 per cent. In fifteen normal rabbits we have obtained an average figure of 3.45 per cent for the liver's proportion of the body weight, with variations ranging from 2.18 per cent to 5.25 per cent. The main mass averaged 72.3 per cent of the total, the ablated caudate 4 per cent, and the right posterior lobe with the caudate stump 23.7 per cent.

#### *Early Changes after Local Portal Occlusion.*

The immediate results of diverting the stream are striking.

Within a minute or so the main liver becomes much smaller, of a deeper purple, and flaccid, whereas the isolated lobe, which now receives all the venous blood, is swollen, tense, and of a rather bright red. In animals dying after 1 or 2 days hemorrhages into spleen, stomach, and small intestines are sometimes found, such as Ponfick observed after removal of the main liver; but, as he also noted, these are infrequent in vigorous animals. The acute passive congestion responsible for them is quickly relieved as the stream bed in the lobe mass is widened through the hypertrophy of the latter.

The mass receiving the portal blood begins to hypertrophy within 3 days by cell proliferation within the lobules, as after ablation of the main liver;<sup>2</sup> and by the end of 12 days the tissue has usually more than doubled, and after 15 days may have trebled. Subsequently its bulk increases more slowly, but eventually reaches that of the entire original liver, and usually surpasses it. Concurrent with the hypertrophy is a progressive atrophy of the mass deprived of portal blood (Figs. 1 and 2).

Owing to reduced capillary distension the lobuli fall together to some extent immediately after the ligation, and within the next few days their cells, which now appear crowded, are noted to have grown smaller, especially near the central vein where the blood supply is poorest. If the animal is weak and dies early, a marked local widening of the capillaries may here be noted, with fine, brown pigmentation of the parenchyma. The condition then is identical with that known in human pathology as the "atrophic red infarct of Zahn." In vigorous rabbits the capillary widening is slight, often absent, and the intralobular atrophy alone attracts attention. The liver mass sometimes dwindle within 12 days to about one-half its original bulk (Table I), though usually the change is slower. Its lobes are flabby, wrinkled, purple, and at this particular period their surface is usually mottled with ill defined, slightly raised, pale spots which may be half a centimeter in diameter (Fig. 3). Several lobuli or parts of them are included in such areas. They are well seen only on the capsular surface, are not degenerative in character, become much more prominent when the blood has partially escaped from the tissues, and are probably areas of relative anemia. The general condition of the liver at this time has much in common with that in dogs during the period of adjustment after an Eck fistula.<sup>3,4</sup> But outspoken degenerative changes are rare in the rabbit liver as compared with the dog, though sometimes a moderate, central fatty degeneration may be noted. The tissue when cut is soft, dark purple, and spleen-like; the lobuli are very small and indistinct. Bile ducts, blood vessels, and interlobular connective tissue are all rendered unusually prominent by the dwindling in parenchyma, and, simulating trabeculæ, add to the spleen-like appearance of the tissue.

Microscopically, one finds many small lobuli to a field, and there is a great increase in the number of cells per unit of surface (Fig. 1), so that the nuclei appear crowded. These last, as well as the cytoplasm, have greatly decreased in bulk, though otherwise they appear unchanged. At the periphery of the lobules a few endothelial cells along the capillaries may be somewhat swollen

<sup>2</sup> Ponfick, E., *Virchows Arch. path. Anat.*, 1895, cxxxviii, suppl., 81.

<sup>3</sup> Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1917, xlvi, 544.

<sup>4</sup> We wish to thank Dr. G. H. Whipple for sections illustrative of the Eck fistula liver.

with granules of a light brown, iron-containing pigment. Such pigmented Kupffer cells become more prominent as time goes on. There is no absolute increase in connective tissue.

### *Late Changes after Local Portal Occlusion.*

The changes up to this point have been partially described by Steenhuis.<sup>5</sup> In his most advanced instance, an animal killed 4½ months after the portal ligation, a further moderate increase in pigmentation and atrophy was noted, but nothing more, a fact difficult to understand save on the assumption that portal collaterals to the main liver had developed, or that the animal was old or in poor condition, all of which factors largely affect the changes. For the atrophy goes further, and quickly too, resulting in a disappearance of all the parenchyma of the main liver, a process sometimes practically completed within 2 months, as we shall show.

Between the 12th and the 40th day after the portal ligation a circulatory readjustment occurs in the dwindling liver mass. Pale spots are no longer seen on its surface, which is of a brighter, more normal red, though tinged with brown. The organ cuts with difficulty, owing to the survival of all its ducts, vessels, and connective tissue, which are brought nearer together by the disappearance of parenchyma; and the tissue disclosed by the knife is more markedly spleen-like, with no trace of a lobular pattern. Histologically, the parenchyma may seem like the normal at first sight, except for the very small size of lobuli and cells, a marked irregularity in the arrangement of the former, and perhaps some irregular capillary widening. Fatty changes are entirely absent, and the atrophy is more evenly distributed. On close scrutiny one perceives here and there parenchymal elements almost without cytoplasm and with small pyknotic nucleus. These scattered cells in the last stages of disappearance are somewhat more numerous toward the center of the lobules. The cell cords in general may be especially atrophic here, and their capillaries wider than at the periphery, additional indications that the central parenchyma suffers most. Pigmented Kupffer cells, distended to a spherical or egg shape, are increased in number, but are found as before only near the periphery of the lobule. The interlobular tissue has nowhere invaded the parenchyma, is absolutely unincreased, and new formed bile ducts are not present. The ducts and vessels, unchanged from their original size, are bent into convolutions as the mass grows smaller, so that their number seems multiplied on cross-section (Fig. 5).

---

<sup>5</sup> Steenhuis, T. S., Experimenteel en kritisch onderzoek over de gevolgen van poortaderafsluiting, Proefschrift Rijks-Universiteit te Gröningen, 1911.

The period required for complete disappearance of the parenchyma varies with the individual (Table I). When the animal is opened the stomach is found to lie in the concavity of the diaphragm, and only on lifting it away is the insignificant remnant of the main liver discovered, with a gall bladder of normal bigness sessile upon it (Fig. 9). The compensating lobe, of great size, extends far down over the abdomen. On nearer inspection, the original lobes of the main liver are found to be represented by three little, flabby, pinkish brown tags (Fig. 4). Cultures from these are sterile. Their surface is roughened by numerous, close packed and tortuous vessels and ducts. Sometimes red varices stud the surface here and there. The hepatic veins, of almost the normal size, at once attract attention as distended cords, grossly disproportionate to the tissue out of which they spring. The hepatic artery, too, is still large. On section the tissue is extremely tough and shows no parenchyma, but everywhere the gaping mouths of vessels and ducts in a slight matrix of connective tissue. The blood immediately above the ligature on the portal trunk is usually fluid, and thrombi are always absent from the branches of the vein.

Microscopically, a few liver cells can be found even when the changes are most pronounced, for the reason that there exist in practically every case a few minute, collateral venules bringing portal blood to the tissue. The rapidity and completeness of the atrophy is in our experiments proportionate to the number of these little collaterals. Their influence may be directly seen where they enter the liver. For example, in Rabbit 27, killed 118 days after the ligation, 2 minute venules were found coursing to small masses of healthy parenchyma in the midst of the atrophy (Fig. 4).

The final changes have much interest. Little by little the lobular units grow smaller and more irregular in form, so that a central vein, when discoverable, may be far off to one side (Fig. 5). There is still no connective tissue invasion or proliferation, but Glisson's capsule becomes increasingly prominent in the picture, appearing to close in on the lobules and envelop them. As the parenchyma grows less, so do its attendant capillaries disappear, and they never survive when the liver cords are gone. Soon there remain only scattered islands consisting of a few small, but healthy looking liver cords with the characteristic capillaries, set in a matrix of connective tissue (Fig. 7). Here and there, near by, three or four parenchymal cells may perhaps be found as an isolated cord, not infrequently with a capillary along one side; and liver cells separated by the en-

veloping matrix of connective tissue may still be recognized. Many such isolated cells have lost their characteristic ground glass appearance, stain a clear pink with eosin, and may be of blunt spindle shape with a relatively large pyknotic nucleus (Fig. 8). Finally parenchymal elements become rare (Fig. 10). Their situation is sometimes indicated by the irregular zone of Kupffer cells distended with pigment that mark the border of the original lobule. As the atrophy increases such elements become prominent, and rounded nests or aggregations of thirty to forty are not infrequent (Fig. 6). Always they are confined to the region of the original periphery of the lobules and are separated from the parenchyma only by the disappearance of the latter. The total absence of invasive tendencies on the part of the connective tissue could not be more clearly shown than by this fact.

The final tag of ducts, vessels, and connective tissue (Fig. 10), representing 50 to 70 gm. of main liver, weighs, in the absence of coccidiosis, cirrhosis, or other intercurrent proliferative change, only 1 to 1.8 gm., which may perhaps be taken as nearly representing the original weight of the non-parenchymal elements. If a cirrhosis was originally present as shown by the caudate sections, the surviving tissue is of greater bulk.

#### *Conditional Character of the Atrophy.*

The liver atrophy in the dog and in man following diversion of the entire portal stream through an Eck fistula is never great. Hence we have questioned whether the complete atrophy observed on local portal diversion in rabbits is inevitable or dependent upon hypertrophy elsewhere. To test the matter hypertrophy has been largely prevented in some animals by tying the bile duct to the lobe mass of the liver after diverting the entire portal stream to it as usual. Under such circumstances the lobe mass undergoes some increase in size through cell proliferation, but combined with this is a continuous, scattered biliary necrosis, and by the end of 12 days a diffuse cirrhosis makes its appearance. After 25 to 30 days the tissue, though still of greater bulk than normal, is indurated and shows microscopically an almost complete replacement with connective tissue. The changes will be more fully described in a later paper. The fact to be emphasized here is that in the absence of hypertrophy of the lobe mass the main liver fails to undergo marked atrophy, although deprived of the portal stream. Such slight atrophy as occurs may be looked upon as inevitable to the circulatory change as such.

The rabbits used were kept under identical conditions and had approximately the same weight. In one series the portal blood was diverted from the main liver and the caudate lobe ablated as usual, while in the other ligation of the bile duct or ducts to the lobe mass was also performed. The local bile stasis had caused no jaundice, and the animals were in good health. The ducts to the posterior lobe, for there may be two or three, lie in an exposed position and can readily be isolated and tied off without damage to their surroundings. From this circumstance, taken with the differing results obtained in the two series of animals, it follows that direct nerve injury can be ruled out as a cause for the complete atrophy after simple portal occlusion. Animals with livers originally abnormal were discarded.

A complicating factor made necessary the early comparison of the series. The progressive cirrhosis occurring when the bile duct from the lobe mass is ligated brings about after a time an obstruction to the portal flow, with chronic passive congestion of the viscera. The spleen enlarges greatly, sometimes becoming cylindrical; and venous collaterals appear rapidly, as a rule preventing marked ascites, though this was once noted. None of the new venous channels had importance for us save such as might enable the portal blood to regain its old stream bed in the main liver; but unfortunately some of this character frequently developed within a few weeks. Usually they followed the course of the veins of Charpy, but sometimes found a way through adhesions. Portal diversion alone not infrequently led to their development in small number. The majority of the rabbits were killed and autopsied prior to their appearance; that is, 12 to 15 days after operation. Such small collaterals to the main liver as were then observed have found place in the general record (Table I). All the animals were killed soon after a feeding.

The weight of the entire liver was somewhat below the normal average in five animals killed 12 days after simple portal occlusion—3.22 per cent of the gross body weight as compared with a normal of 3.45 per cent. Every individual weight, though, was within the normal range of 2.18 to 5.28 per cent. In the five rabbits with local bile stasis added to portal ligation, whereby parenchymal destruction was superimposed upon hypertrophy, the livers weighed more, averaging 3.62 per cent of the gross weight. In the animal with simple atrophy, and its companion with bile stasis, killed after 15 days, the weights were 4.5 and 5.78 per cent of the gross, respectively. The average after longer periods was 3.73 per cent for ten rabbits killed from 21 to 68 days after portal diversion, and 3.69 per cent for three animals with an additional bile stasis, examined after from 21 to 30 days. It would seem that the reparative changes went beyond a mere replacement; as

TABLE I.  
*Liver Changes Following Diversion of the Portal Stream to the Lobe Mass.*

Rabbit No.	Body weight. At operation.	Collateral veins to liver. Final weight.	Liver weight.			Liver's per cent of body weight.			Condition of main liver. Lobe mass.	Remarks.
			Entire liver. mass.	Main liver. mass.	Lobe mass.	Entire mass	Main liver.	Lobe mass.		
Duration of experiment, 12 days.										
1	2,200	2,475	None.	72.4	28.3	44.1	3.62	1.15	1.78	Marked atrophy; many pale spots.
2*	2,050	2,175	One of 1½ mm.	78.6	43.3	35.3	3.62	1.99	1.63	Little atrophy; no spots.
3	2,150	2,050	One of ¼ mm.	75.4	39.8	35.6	3.68	1.94	1.74	Marked atrophy; many spots.
4	2,150	2,050	One of 2½ mm.	76.6	48.0	28.6	3.74	2.34	1.4	Little atrophy; no spots.
5	1,900	1,600	One of 3 mm.	46.4	21.7	24.7	2.9	1.36	1.54	Marked atrophy; no spots.
6	1,925	1,600	Two of 1 mm.	65.2	30.2	32.8	4.07	1.89	2.05	Little atrophy; no spots.
7	1,525	One of 1 mm. and several smaller.	48.8	16.8	32.0	3.2	1.1	2.1	Marked atrophy; some spots.	
8	1,850	1,700	None.	55.0	29.9	25.1	3.24	1.76	1.48	Marked atrophy; many spots.
9	1,550	1,275	One of 1½ mm.	43.1	23.2	19.9	3.38	1.83	1.55	Marked atrophy; many spots.
10	1,750	1,650	One of ½ mm.	56.4	38.3	18.1	3.42	2.32	1.1	Slight atrophy; some spots.

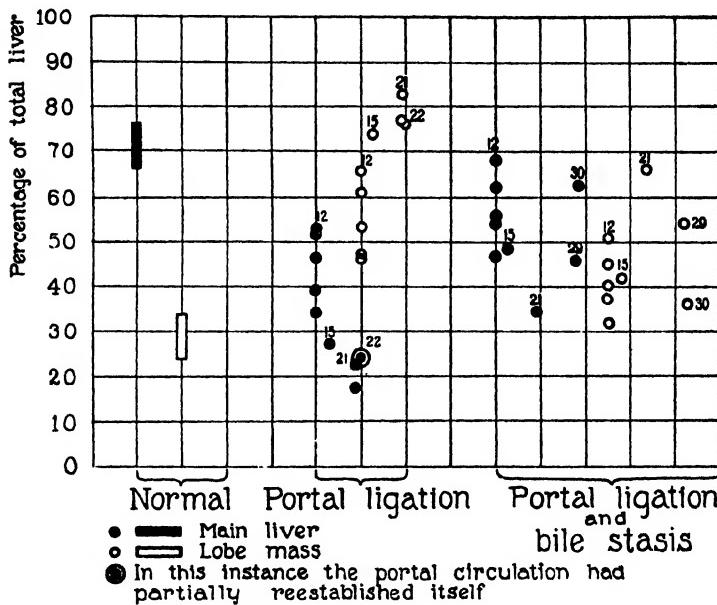
Duration of experiment, 15 days.										
11	1,425	1,525	One of 1 mm.	70.2	18.6	51.6	4.6	1.22	3.38	Marked atrophy; some spots.
12	1,425	1,400	Two of 1 mm.	80.9	38.9	34.2	5.78	2.78	2.44	Slight atrophy; no spots.
										Of total liver weight 7.8 gm. is caudate stump in simple hypertrophy.
Later periods.										
days										
13	21	2,300	2,250	Only very fine ones.	73.5	17.0	56.5	3.27	0.76	2.51
14	29	2,175	2,300	Only very fine ones.	78.5	37.0	41.5	3.42	1.61	1.81
15	30	2,275	2,275	Numerous very fine ones; also one of 1 mm. and of 2 mm.	69.1	44.0	16.4	3.02	1.94	0.72
16	22	1,825	1,900	One of 5 mm.	122.7	30.5	92.2	6.47	1.61	4.86
17	21	1,875	1,375	One of 2 mm.	68.7	22.8	45.9	5.0	1.66	3.34
18	21	1,575	1,750	None (?)	55.4	9.3	46.1	3.17	0.53	2.64
19	21	1,675	1,950	None. Not noted.	77.5	15.2	62.3	3.98	0.78	2.2
20	25				73.5	19.5	54.0		"	"

\* The findings in bold faced type relate to animals in which the bile duct of the lobe mass was also ligated.

TABLE I—Concluded.

Rabbit No.	Body weight. At oper. Final weight.		Collateral veins to liver.		Liver weight.		Liver's per cent of body weight.		Condition of main liver.	Remarks.
	days	gm.	days	gm.	Entire liver. mass.	Main liver. mass.	Lobe mass.			
Later periods.										
21	55	2,125	2,550	One of $\frac{1}{2}$ mm.	84.3	5.0	79.3	3.31	0.2	Advanced atrophy.
22	58	2,025	2,250	Two " 1 "	74.1	10.1	64.0	3.3	0.46	" "
23	64	2,150	One " $1\frac{1}{2}$ "	59.9	6.7	53.2	2.79	0.31	" "	
24	65	2,050	2,050	" 1 " several hair-like.	70.9	1.8	69.1	3.46	0.09	Practically complete atrophy.
25	68	2,025	2,050	" of 1 mm.	79.3	4.0	75.3	3.87	0.2	Almost complete atrophy.
26	104	2,250	2,300	None.	82.4	9.4(1)	73.0	3.58	0.41	Advanced atrophy.
27	118	1,825	2,375	Two of $\frac{1}{2}$ mm.	99.0	1.4	97.6			Unusual delay in atrophy.
28	185	2,400	" " $1\frac{1}{2}$ " and 1 mm. respectively.	71.1	1.6	69.5	2.96	0.07	Liver weighed with blood retained.	

with other tissues. The idea is borne out by the decrease in size of the liver occurring later. In seven animals killed 55 to 185 days after portal diversion the liver averaged only 3.22 per cent of the body weight.



TEXT-FIG. 1. The change in relative proportions of main liver and lobe mass after local portal diversion with and without ligation of the bile duct of the mass receiving the portal stream (see Table I). The range of the normal proportions, as observed in thirteen rabbits, is given in the short columns. The caudate lobe had not been ablated in these instances as in the case of the operated individuals. The results in the latter are given in dots, and the number of days elapsing after operation is indicated in small numerals. When there is but one of these above a vertical row of dots it is supposed to apply to all.

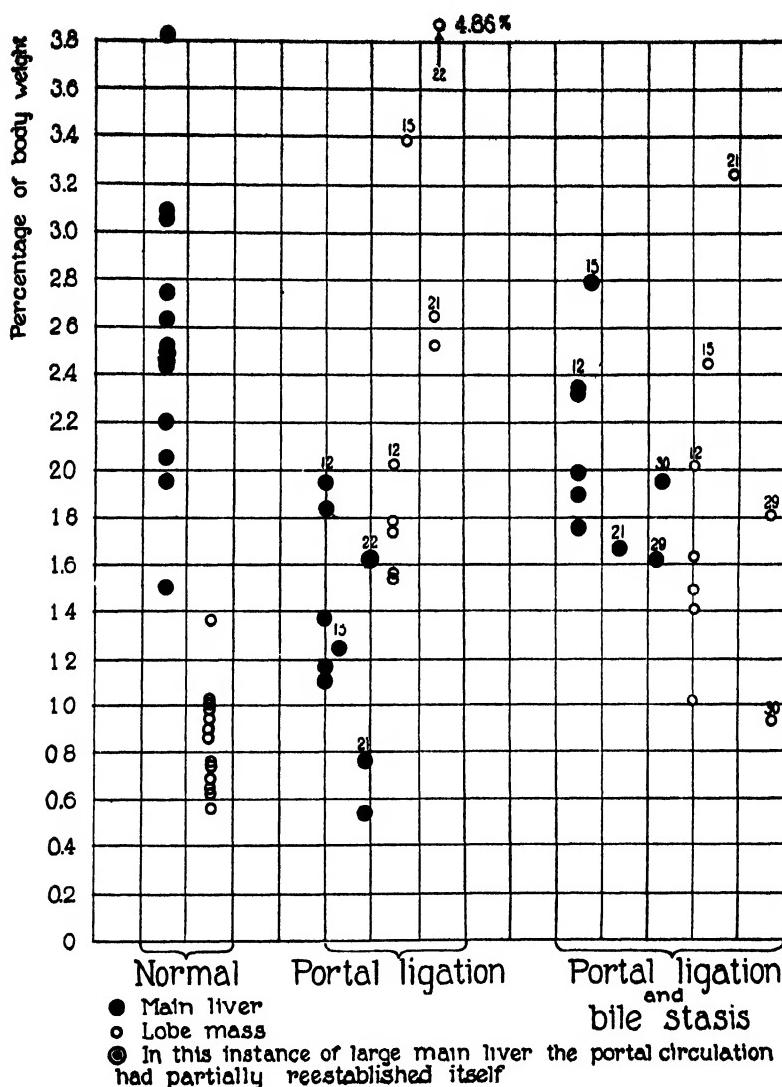
The distribution of the hepatic parenchyma was very different in the two series of animals (Text-fig. 1). Under normal conditions the relative size of the liver masses does not vary much, the larger ranging from 75.7 to 66.3 per cent and the smaller from 24.3 to 33.7 per cent of the whole. After operation not only did the general relations

change but the individual variations in the liver masses became greater. The latter alteration has no interest for us, however. The important point is that the main liver retained far more of its parenchyma after diversion of its portal blood if progressive hypertrophy of the lobe mass was prevented by biliary stasis (Text-fig. 2). The fact is illustrated by differences in both the proportional and actual weights of the liver masses; and the longer the period after operation the greater was the disparity noted, granting the absence of large hepatopetal collaterals. After 4 weeks of portal diversion plus bile stasis the main liver was sometimes three times as big as after but 3 weeks of uncomplicated portal diversion (compare Nos. 14 and 18, Table I, and see also Text-fig. 2).

The importance of portal venules measuring 1 to 2 mm. in diameter for the maintenance of the large mass of liver tissue that remains shortly after operation is negligible, as the general records show (Table I). The animal with the largest collaterals after 12 days, Rabbit 5, the subject of simple portal diversion, had a liver atrophy far greater than occurred in any of the controls with almost no collaterals but with bile stasis. Even at a late period of atrophy, when the liver is greatly shrunken, a collateral of 1 mm. diameter suffices to maintain but a small portion of parenchyma, as we have repeatedly found (Fig. 4). Yet the importance of very large collaterals is not to be gainsaid. It is well seen in the case of Rabbit 16, Table I.

A number of special instances excluded from the table and the text-figures for good cause might be quoted to illustrate the dependence of the pronounced liver atrophy on a compensating hypertrophy. One will suffice.

In a rabbit weighing 1,600 gm., killed 12 days after simple portal diversion, an unexpectedly large main liver was found. It weighed 33.2 gm., representing thus 2.1 per cent of the body weight of the animal, a proportion met with in other animals only when local bile stasis had prevented hypertrophy of the lobe mass. There were no collaterals to explain the condition, but in the lobe mass, which weighed only 22 gm., an atrophy of unknown cause was encountered, affecting at least half of its original tissue content, while the remainder was hypertrophied.



TEXT-FIG. 2. Changes in weight of the main liver and lobe mass, as expressed in percentages of the gross body weight. The number of days elapsing after operation is given in small numerals as in Text-fig. 1.

*Functional Activities of the Atrophic Tissue.*

The functional activities of an hepatic tissue deprived of portal blood and competing with a hypertrophic parenchyma that receives the entire portal stream have much interest. Asp<sup>6</sup> showed long ago that the immediate effect of a local portal occlusion in the rabbit is to lessen secretion from the affected parenchyma. In our animals the atrophic tissue still secreted bile in good quantity at a period when the liver mass possessing a monopoly of the portal stream was far advanced in compensatory hypertrophy.

The biliary secretion of the rabbit is thin and copious—5 cc. per kilo of animal in 1 hour according to Heidenhain,<sup>7</sup> or 169 gm. per kilo of liver. Krause<sup>8</sup> obtained an average of 115.7 gm. of bile per kilo of animal in 24 hours. The weight of his animals was taken after the intestinal contents had been removed, which entailed a reduction of some 20 per cent from the gross weight. The quantity of rabbit bile varies greatly from hour to hour; the pigments fail to give the ordinary color reactions in a satisfactory way; and neither they nor the other bile constituents have been sufficiently studied to establish a norm. For these reasons we have merely observed the amount and color of the secretion from liver masses in atrophy and submitted it to Pettenkofer's test, while well aware that the latter gives positive results with other substances than bile salts, notably with cholesterol.

*Method.*

Two operations were necessary, the first to ligate the portal vein to the main liver, and the second, weeks or months later, for the ligation of the bile duct to the atrophic mass and the production of a fistula. The duct was twice tied above the point of entrance of the branch from the compensating lobe mass; the gall bladder was slit at its tip; a fine, rubber-covered catheter was introduced; and this last was sewed to the lip of the abdominal wound in such fashion that the atrophic liver was left in its usual position. The gall bladder and larger bile passages above the ligatures were gently and repeatedly flushed with salt solution injected and withdrawn through the cannula. This was done to wash out all traces of the bile previously reaching the gall bladder from the hypertrophic tissue. The washing never caused even a temporary cessation of the bile flow so far as could be judged from cases in which this was abundant. The cannula was now connected with a thick walled, rubber tube that led to a sterile, flat bottle strapped to the abdomen with adhesive. Through the stopper an air-vent tube was carried around to the animal's back under a many tailed bandage. The

<sup>6</sup> Asp, G., *Arb. physiol. Anstalt Leipzig*, 1873, viii, 124.

<sup>7</sup> Heidenhain, R., in Hermann, L., *Handbuch der Physiologie*, Leipsic, 1883, v, pt. 1, 252.

<sup>8</sup> Krause, W., *Die Anatomie des Kaninchens*, Leipsic, 2nd edition, 1884.

bottle was adjusted at such an angle that the bile dropped into it from the end of the tube. The animals tolerated the arrangement well, and sometimes bile collection was continued for a period of 48 hours.

The several experiments in which bile collections were made from main livers in a state of moderate atrophy need not be cited in their entirety. In all an unexpectedly large amount of bile was obtained.

For example, 15 cc. of bright green bile giving a marked Pettenkofer reaction was obtained in 18 hours from an atrophic main liver weighing 19.5 gm., in the presence of a lobe mass of 54 gm. At the time when the bile fistula was produced, 25 days after portal closure, the original ligature on the vein was found to have relaxed, allowing a slight leak to the main liver, and accounting for the slightly delayed atrophy. A second, and completely occluding, ligature was laid on prior to the collection of bile. The latter was secreted at a rate much below the normal, though still considerable—43 cc. per hour per kilo of the atrophic tissue.

More interest attaches to instances in which the compensating mass approximated the whole original liver in size, and presumably in function, since the atrophic main liver had become very small.

Rabbit 24 had on the 65th day after operation a main liver weighing but 1.8 gm. and a compensating mass of 65.1 gm. On the day previous, while the animal was in excellent condition, the main bile duct was ligated and a fistula produced as usual. The ligature was placed above the entrance into the main duct of the branch from the left posterior lobe, and in consequence the secretion from only about two-thirds of the atrophic mass was obtained. At autopsy no portal collaterals to this fraction were found, nor was there the least biliary obstruction. The gall bladder was empty. From it 0.8 cc. of clear, watery fluid had come away in 23½ hours. This had a faint greenish tinge and gave a faint Pettenkofer reaction. The microscope later showed that definite islands of liver parenchyma were present in the tissue furnishing the bile.

A similar duct ligation with the branch from the left posterior lobe excluded was done in Rabbit 23 with a main liver of 6.7 gm., and 53.2 gm. of compensating tissue 64 days after operation. The atrophic tissue from which bile was collected, some two-fifths of the whole main liver, received a portal collateral 1.5 mm. in diameter. Nevertheless, there was secreted from it only 0.9 cc. of bile in 21 hours, though this was medium green in color and gave an outspoken Pettenkofer reaction. The tissue that was drained still consisted predominantly of parenchyma.

Rabbit 21 had a main liver of 5 gm. and a compensating mass of 79.3 gm. on the 55th day after operation. The main duct was ligated successfully, but a blood clot stopped the cannula, so the animal was killed 4 hours after the operation. During this period it had been lively. The stomach was full of food. Only 0.5 cc. of fluid, and this faintly green, was present in the collapsed gall bladder. No obstruction was found to the flow of bile through the ducts; and the

main liver mass was still predominantly parenchymal. A single hepatopetal portal collateral existed, 0.5 mm. in diameter.

The amount of parenchyma present in the atrophic main liver of Rabbit 24 was small. In the other two instances, though, where there was more of it, the amount of bile was far below that called for on calculation, had the conditions been normal, after allowing for 1.8 gm. of scaffolding, ducts, and vessels in the atrophic mass. Furthermore, the secretion in two of the three animals was markedly deficient in pigment, and in one gave but a faint Petterkofer reaction. Yet there is no doubt that the hepatic tissue, even when extremely atrophic, does manufacture bile of a sort, and is not prevented from so doing by the presence of a compensatory liver mass of very large size. It should be remembered in this connection that the atrophic tissue receives through its large hepatic artery a liberal supply of blood.

Glycogen was sought with Best's carmine stain in three instances of far advanced atrophy. The method is subject to some errors, as Rusk<sup>9</sup> has brought out, yet it seemed preferable to a chemical analysis because of the greatly altered proportion of parenchyma in the tissue. The preparations showed a practically identical amount and distribution of glycogen in the hypertrophic and atrophic parenchyma of the same individual, even in cases as advanced as Nos. 24 and 25 (Table I). Neither the competition of the hypertrophic mass nor its favorable situation on the portal stream was sufficient to deprive the main liver of even relatively little glycogen. But this is scarcely surprising when one considers how widely the substance is distributed in the body, and that dextrose is normally present in the arterial blood. An interesting aspect of the findings is the evidence they give for the belief that such liver parenchyma as survives atrophy to a late period remains in remarkably good condition. For the glycogen content of unhealthy tissues is usually greatly altered.

#### DISCUSSION.

##### *Conditional Atrophy in the Dog.*

Recently one of us, with Dr. Philip D. McMaster, has ligated the portal trunk to the three upper lobes of the liver in a number of dogs. Changes ensued much more slowly than in the rabbit and are not yet

<sup>9</sup> Rusk, G. Y., *Univ. California Pub. Path.*, 1912, ii, 83.

complete, after 3 months, but the tissue deprived of portal blood has diminished to less than one-third of its original bulk through a simple atrophy, with a corresponding hypertrophy elsewhere.

### *Review of the Literature.*

Previous work on the result of local portal occlusion has been well summarized by Winternitz,<sup>10</sup> who himself treats of the early changes in human livers. Though some observers, Solovieff<sup>11</sup> and others, have claimed that the occlusion of portal branches leads to cirrhosis, and Ehrhardt<sup>12</sup> stated that a moderate atrophy without cirrhosis ensues, the prevailing view, long since crystallized, is that no liver changes occur either in man or the laboratory animals unless pressure is abnormally low in the hepatic artery or high in the vena cava as the result of a disturbed systemic circulation. Under such contributing circumstances one finds within a few days the so called red infarct of Zahn in the region deprived of portal blood. Here the lobular capillaries are much distended, presumably from a venous stasis, and the liver cords somewhat atrophied. In the gross the liver portion is dark red and slightly sunken. The late changes have not been described, according to Winternitz, for the reason that such diseases as produce embolus or thrombosis in the portal system almost always end fatally within a brief period.

It is interesting to note here and there in the literature isolated statements that confirm our findings, and like them indicate that the prevailing view as just given is erroneous. Thus Frerichs<sup>13</sup> stated in 1858 on the basis of his own observations that local portal occlusion leads to parenchymal atrophy with liver scarring. According to Ehrhardt,<sup>12</sup> Nauwerck saw a case in which the left lobe of the liver was diminished to the size of the fist, with compensatory hypertrophy of the right lobe, as a result of long standing occlusion of the left portal branch. Ehrhardt himself produced a moderate atrophy with compensatory hypertrophy by local portal ligation in cats, but he did not follow the changes long.

As already mentioned, most authors state that the development of a red infarct of Zahn after local portal occlusion is conditional upon a general circulatory disturbance. The fact that Zahn<sup>14</sup> himself produced typical red infarcts by injecting mercury into a mesenteric vein of otherwise healthy dogs seems to have been forgotten, as has also his view that the condition is an atrophy from inactivity owing to the lack of portal blood, combined with a pressure atrophy

<sup>10</sup> Winternitz, M. C., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 396.

<sup>11</sup> Solovieff, A., *Virchows Arch. path. Anat.*, 1875, lxii, 195.

<sup>12</sup> Ehrhardt, O., *Verhandl. deutsch. Ges. Chir.*, 31 Kong., 1902, xxxi, 544.

<sup>13</sup> Frerichs, F. T., *Klinik der Leberkrankheiten*, Brunswick, 1858 (Sydenham Society's translation, A clinical treatise on diseases of the liver, London, 1861).

<sup>14</sup> Zahn, F. W., *Verhandl. Ges. deutsch. Naturforsch. u. Aerzte*, 69 Versamml., 1897-98, ii, pt. 2, 10.

from stasis as the result of retrograde pressure in the hepatic veins. The drawing given by Chiari<sup>15</sup> shows a histological condition identical with that present in our rabbits 12 to 15 days after the ligation. There can be no doubt that a general circulatory derangement renders the atrophy more prominent, as was the case in some of our animals that fell sick. The widening toward the central vein of the lobular capillaries, when marked, is doubtless often the result of retrograde pressure; but it occurred in moderate degree in some of our well conditioned rabbits and would seem then to have been merely the consequence of rapid atrophy of the liver cords. That there may occur complete parenchymal atrophy which is conditional on hypertrophy of the remaining parenchyma has not been realized heretofore.

Since the completion of our work the paper of Steenhuis<sup>6</sup> has come to attention. It has attracted little notice among pathologists, owing perhaps to the fact that its author laid stress rather upon the surgical implications of his findings than upon the pathological. But Steenhuis ligated the portal trunk to the main liver of the rabbit, just as we have done, and observed an atrophy of medium grade thereafter, with the development of pigmented Kupffer cells. He did not follow the changes to even approximate completion, since in his most advanced instance a considerable bulk of liver tissue still remained, as the pictures and description clearly show. He noted the influence of portal collaterals to check the atrophy and drew the conclusion, since proved erroneous, that a direct portal stream is essential to liver survival.

#### *Physiological Considerations.*

Several reasons can be suggested for the changes which follow a local diversion of the portal stream. Among them are the following:

(a) *Direct Influence of the Altered Circulation.*—By the ligation of its portal trunk the main liver is supplied solely with arterial blood, which latter may be so unsuited to the liver cells that they can survive and function only in the absence of competition, as under the circumstances of an Eck fistula. The high oxygen content of the blood can scarcely be invoked as a cause of the hypothetical unfitness, since the atrophy is least at the periphery of the lobules where oxygenation is greatest.

(b) *Altered Functional Opportunities.*—By local portal obstruction one portion of liver tissue is deprived of its normal opportunity to obtain many substances and must compete with another receiving them in undue quantity. Functional atrophy and hypertrophy should follow as a matter of course. The extent and rapidity of the

<sup>15</sup> Chiari, H., *Z. Heilk.*, 1898, xix, 475.

changes alone are surprising. The hypertrophy goes on almost, perhaps quite, as rapidly as if the tissue deprived of portal blood had been ablated.

Few rabbits survive the abrupt removal of the main liver. Ponfick's instances do not enable one to judge when the compensating hypertrophy was complete. Von Meister<sup>16</sup> states that the right posterior lobe and caudate attain the weight of the whole liver in from 45 to 60 days. But this weight he puts at only 2.91 per cent of the gross body weight, whereas 3.45 per cent is nearer the truth and has been the basis of our calculations. In one of our cases such a proportion to the body weight was actually attained by the hypertrophied mass within 65 days and in another within 68, while the functional adequacy of the tissue was attested by complete atrophy of the main liver in one instance and approximately complete atrophy in the other. These results become more striking when one considers that our animals were adults weighing 2,000 gm., whereas von Meister's were young and of 900 to 1,400 gm., that is, far more favorable to hypertrophy, as he showed; and when the further fact is added that the caudate lobes were cut away in our cases so that there was less tissue capable of hypertrophy. Nasse<sup>17</sup> found that 4 months was required for the disappearance of the main liver mass of the rabbit after ligation of its bile duct.

If the rate of the hypertrophy is approximately the same after local portal deprivation as after local ablation, this might mean either that the tissue deprived of portal blood is useless to the organism or that hypertrophy goes on irrespective of its activities. The pros and cons cannot be profitably discussed, but both alternatives entail the assumption that the liver is wholly a portal organ, finding its reason for being in the substances carried to it on the portal blood and in them only. The biliary activity of the atrophic tissue does not constitute evidence against such a view, even granting that the substances from which bile pigment is produced come to the liver normally on the portal blood alone—an assumption yet to be proved. The small hepatic mass which receives the entire portal blood after local diversion of the stream must be thought of as unable to cope with its functional opportunities for some time, so that much material for liver activity passes through into the general circulation and reaches the atrophic competing mass. Later, as the compensating tissue attains the size and functional power of the whole original liver, less of the portal material may be supposed to escape through it. Yet

<sup>16</sup> von Meister, V., *Beitr. path. Anat. u. allg. Path.*, 1894, xv, 1.

<sup>17</sup> Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, 525.

that a portion does pass through the normal liver has been shown by Van Slyke<sup>18</sup> for the amino-acids. It is perhaps a similar passage of material that enables a liver remnant wholly deprived of portal blood and advanced in atrophy to continue the formation of bile pigment, albeit in reduced quantity.

(c) *Food Deprivation.*—Though the main liver mass of our animals undoubtedly received relatively little of some at least of the substances upon which it normally acts, this need not have been the essential cause of its atrophy. Perhaps a special food is essential to liver maintenance. Whether this comes from a systemic or portal source, the tissue receiving the whole portal stream would have the best opportunity at it, and, possessing the superior powers of a growing, healthy tissue, would gradually increase its rival's deprivation. But were the source of the hypothetical food substance systemic, not portal, one would expect atrophy and hypertrophy to go on more slowly than is the actual case and perhaps not to reach completion. The question whether food substances can be distinguished from those utilized in function need not be entered upon.

Much of the foregoing incomplete analysis is only warranted in as far as it illustrates the vital importance for the liver tissue of a position on the portal stream. A situation there is not obligate, it is true. For the liver deprived of the direct portal stream by an Eck fistula still survives,—though perhaps only because it still receives the portal substances, at one remove, so to speak, by way of the systemic circulation.

#### *Influence on Liver Development.*

Toldt and Zuckerkandl<sup>19</sup> demonstrated in 1876 that the normal human liver undergoes notable changes in shape during the period from birth to adult life. In some portions of the organ atrophy occurs, while in others there is hypertrophy. The atrophy seems to be identical with that after portal diversion, while, when it is complete, as not infrequently happens, there are left behind the same large corded ducts and blood vessels. Toldt and Zuckerkandl attribute the changes to pressure from the surrounding organs and adduce

<sup>18</sup> Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

<sup>19</sup> Toldt and Zuckerkandl, *Sitzungsber. k. Akad. Wissensch. Wien., 3te Abt.*, 1876, lxxii, 241.

reasons for their belief. We would go a step further and suggest that the transmitted pressure may produce its effect in some instances through local alterations in the portal stream.

Mall,<sup>20</sup> who confirms the findings of Toldt and Zuckerkandl, points out in another connection that the distribution of the portal blood to the hepatic parenchyma is in general remarkably even, as can be demonstrated by injection methods. No hepatic region is specially favored. In the light of our observations the necessity for this is clear. For any enduring local irregularity in the portal flow will result in a shift of parenchyma. One of the commonest shifts observed by Toldt and Zuckerkandl entailed a complete atrophy of the left lobe. Herringham<sup>21</sup> found ten such cases in 3,000 autopsies. A reason for this is not far to seek, nor for the rarity of atrophy of the right lobe. In man the right branch of the portal vein is extremely short and thick, breaking up almost at once into many lesser vessels; whereas the left branch courses for a long distance through the parenchyma as a single slender trunk, much exposed to transmitted pressure.

#### *Bearing on Liver Lesions.*

Pathologists have long recognized that liver destruction frequently induces a local compensatory hypertrophy. From our observations it is evident that there exists, conversely, a type of destruction dependent upon compensatory hypertrophy. The knowledge should aid in an understanding of certain chronic liver lesions. The advanced local atrophy sometimes occurring in livers containing an echinococcus cyst, a gumma, slow growing tumor, or other limited process may well be the result of pressure upon portal radicles. In such instances there is present elsewhere in the organ an abundance of parenchyma capable of compensatory proliferation. The opportunities for marked changes are far less favorable in the atrophic cirrhosis of Laennec. The irregular stenosis and occlusion of portal branches which characterize the disease fail to lead to a complete atrophy of large liver portions because the parenchyma which under ordinary circumstances would proliferate in compensation is prevented from so

<sup>20</sup> Mall, F. P., *Am. J. Anat.*, 1906, v, 227.

<sup>21</sup> Herringham, W. P., *St. Bartholomew's Hosp. Rep.* 1905, xli, 15.

doing by a confining connective tissue. However, numerous small areas of partial hypertrophy and atrophy may and do exist.<sup>22</sup> In syphilitic livers with sharply localized scarring no such impediment is present; and we would suggest that local portal obstruction is a prime cause for the extreme atrophy and hypertrophy which in such cases frequently lead to great hepatic distortion. According to Sternberg<sup>23</sup> a whole lobe of the syphilitic hepar lobatum may be reduced to a connective tissue appendage.

The disappearance of large masses of liver tissue without the least connective tissue replacement may take place in the very old. According to MacCallum<sup>24</sup> whole layers of parenchyma may disappear, . . . "on the surface of the organ blood-vessels, bile-ducts, and the fibrous skeleton of the liver lie exposed." Not impossibly the atrophy is one of deprivation, and identical in its essentials with that resulting from local portal occlusion.

#### SUMMARY.

The occlusion of portal branches to a part of the liver of the rabbit leads to a progressive and ultimately complete atrophy of the parenchyma in the region deprived of portal blood, and to hypertrophy of the rest of the hepatic tissue which receives such blood in excess. Three-fourths of the liver may thus be reduced to a fibrous tag within 2 months, while the remaining fourth attains the bulk of the entire original organ. The atrophy is simple, unaccompanied by obvious degenerative changes or by any connective tissue replacement. More important, it is conditional in nature, failing to progress when the bile duct from the proliferating tissue is ligated and its hypertrophy checked in this way.

There are indications in the literature that an atrophy conditional on hypertrophy, such as is here described, occurs in man after local portal occlusion. And some experiments in our laboratory, not yet completed, show definitely its occurrence in the dog. The changes take place slowly in the canine liver. After 3 months the tissue de-

<sup>22</sup> MacCallum, W. G., *J. Am. Med. Assn.*, 1904, xliii, 649.

<sup>23</sup> Sternberg, C., in Aschoff, L., *Pathologische Anatomie*, Jena, 1911, ii, 855.

<sup>24</sup> MacCallum, W. G., *Text-book of pathology*, Philadelphia and New York, 2nd edition, 1914, 60.

prived of portal blood has diminished to about one-third of its original bulk. The conditional character of the atrophy is proven by its failure to occur to any similar degree in the absence of a compensating parenchyma, as when the portal stream is diverted from the whole liver by way of an Eck fistula.

Is the atrophy functional? If so, its completeness would indicate that the liver has no essential activity—none on which its maintenance depends—that it is not intimately connected with substances derived from organs drained by the portal system. Observations on the rate of hypertrophy after local diversion of the portal stream and on the character of the bile secreted by the atrophic tissue may be taken to favor such a view. The hypertrophy is nearly, perhaps quite, as rapid as if the tissue deprived of portal blood had been removed from the body. The bile secreted from a liver mass far advanced in atrophy and competing with a large bulk of parenchyma that receives the entire portal stream is almost colorless and may give but a weak Pettenkofer reaction. Glycogen, on the other hand, is present in the atrophic cells in approximately the same amount and distribution as in the hypertrophic liver tissue of the same animal.

The fact that a parenchymal shift follows local disturbances in the portal stream has a bearing on the cause of certain alterations in the shape of the normal liver that have been loosely attributed heretofore to pressure from the surrounding organs. It also has some interest in connection with pathological changes. Liver hypertrophy dependent on a preceding destruction has long been known to pathologists. Now a type of destruction dependent on compensatory hypertrophy must also be reckoned with. The occurrence of changes of the latter character will explain certain of the lesions observed in diseases that involve a disturbance of the portal flow to portions of the liver substance.

#### EXPLANATION OF PLATES.

##### PLATE 67.

Figs. 1 and 2. Rabbit 7, Table I. Hepatic atrophy and hypertrophy, respectively, 12 days after diversion of the portal stream from the main liver. Three lobules of the main liver are barely equal in size to one of the hypertrophic lobe mass. The cells in atrophy are smaller and appear crowded together. Hematoxylin and eosin.

## PLATE 68.

FIG. 3. Rabbit 9, Table I. Pale spots on the surface of a main liver 12 days after the ligation of its portal trunk. To the right is seen the corresponding hypertrophic lobe mass.

FIG. 4. Rabbit 27, Table I; 118 days. The effect of portal collaterals. The main liver is in complete atrophy save for a small, button-like area of healthy looking parenchyma, which receives at its center a portal venule from the lesser curvature of the stomach. Fig. 9 is a photograph of the entire specimen, and Fig. 10 shows the microscopic findings in the main liver.

## PLATE 69.

FIG. 5. Rabbit 21, Table I; 55 days. Advanced atrophy of the main liver. The parenchyma is greatly diminished in amount, and the surviving lobules are extremely irregular. Bile ducts and blood vessels are prominent and numerous, and the intralobular capillaries are much widened in this special instance, but the lobules themselves are uninhabited by connective tissue which, however, is definitely increased. The increase was present prior to operation, as the liver fragment taken at the time shows. The dark rounded masses here and there are Kupffer cells distended with pigment. Hematoxylin and eosin.

FIG. 6. Rabbit 25, Table I; 68 days. Another instance of advanced atrophy of the main liver, but with pigmented Kupffer cells in unusual abundance. The peculiar appearance of the liver cells is due to the fixative. The great number of bile ducts relative to parenchyma should be noted, as also the characteristic absence of any increase in connective tissue, save for a slight thickening about the ducts, that was present prior to operation. Hematoxylin and eosin.

## PLATE 70.

FIG. 7. Rabbit 24, Table I; 65 days. A late stage of parenchymal disappearance. Two small islands of liver cords with characteristic capillaries can be discerned. Many ovoid, pigmented Kupffer cells are present here and there. Methylene blue and eosin.

FIG. 8. A highly magnified parenchymal island from the same specimen. Near it are individual liver cords and cells isolated by the enveloping connective tissue. To the right and left lie Kupffer cells distended with pigment. Methylene blue and eosin.

## PLATE 71.

FIG. 9. Rabbit 27, Table I; 118 days. Practically complete atrophy of the main liver, with compensatory hypertrophy of the lobe mass. In Fig. 4 a nearer view of the main liver is given.

FIG. 10. Condition of the main liver in the same rabbit. Save for an occasional cell, which cannot be discerned in the picture, the parenchyma is entirely gone. There remain arteries, veins, and bile ducts in a slight matrix of connective tissue, with some aggregations of round cells. Eosin and methylene blue.

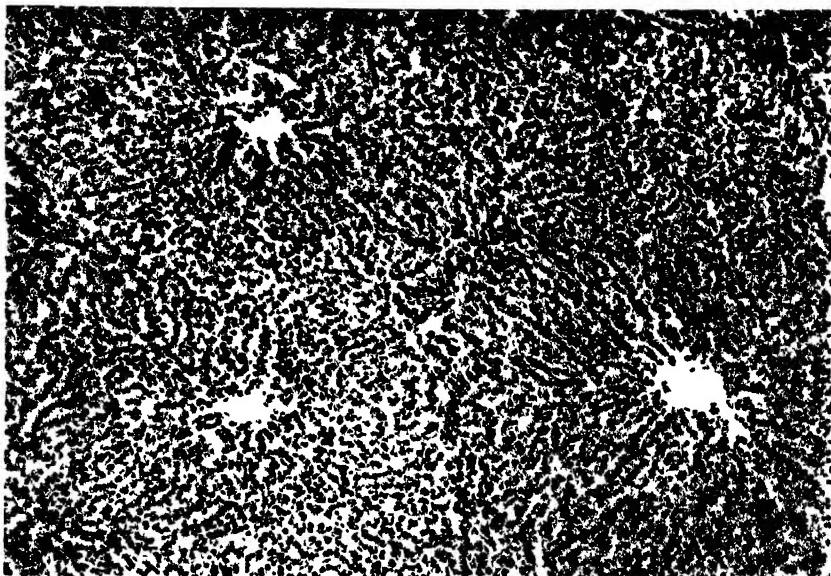


FIG. 1.

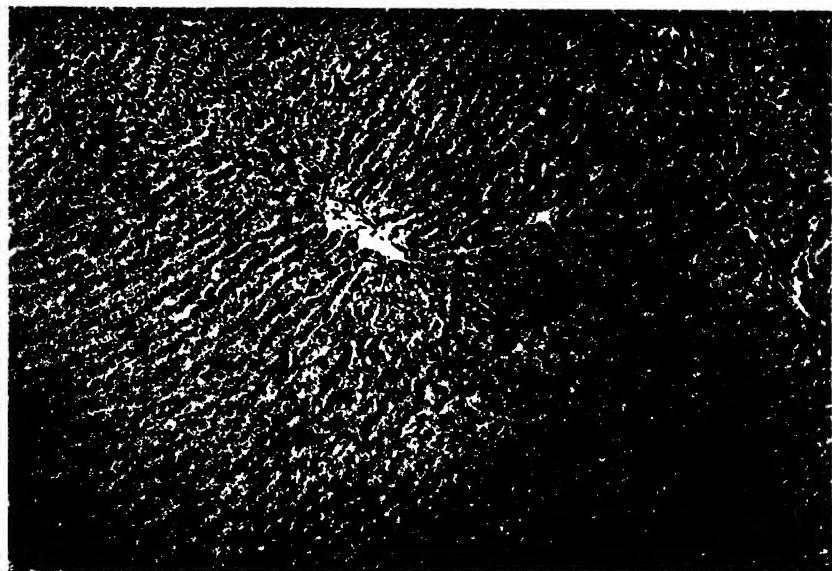


FIG. 2

(Rous and Larimore. Portal blood and liver maintenance.)





FIG. 3.



FIG. 4.

(Rous and Larimore: Portal blood and liver maintenance.)



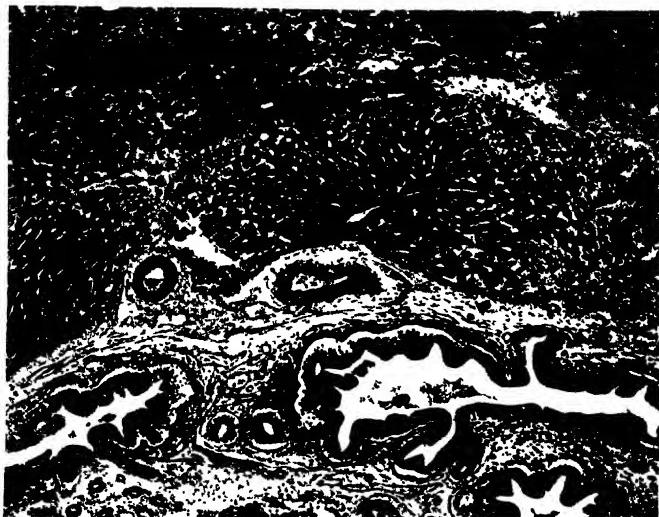


FIG. 5

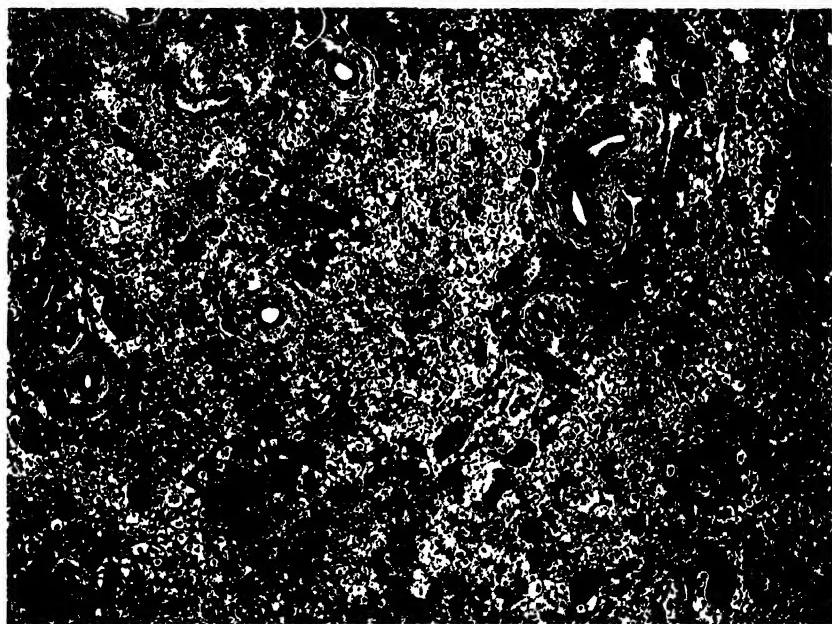


FIG. 6.

(Rous and Larimore: Portal blood and liver maintenance.)



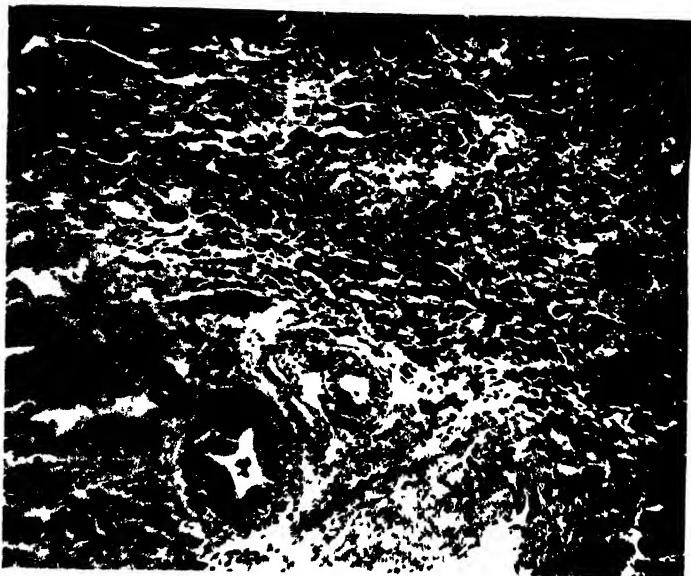


FIG. 7.

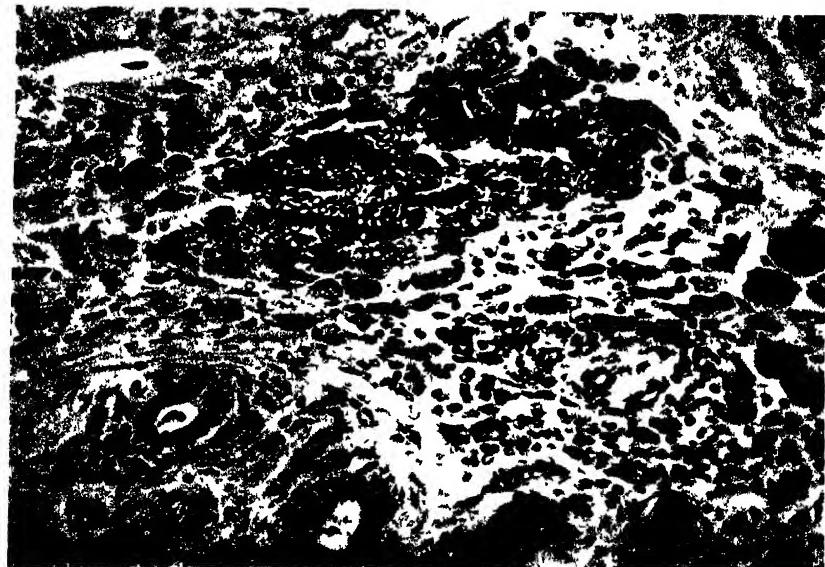


FIG. 8.

(Rous and Larimore: Portal blood and liver maintenance.)



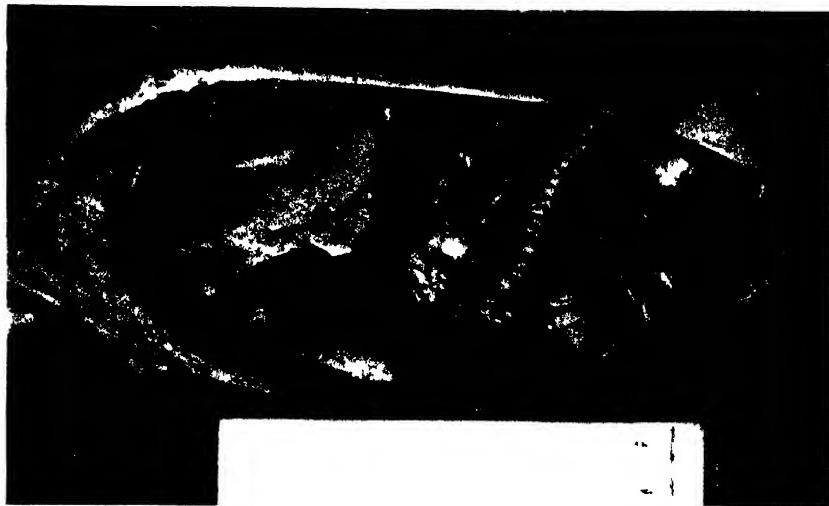


FIG. 9

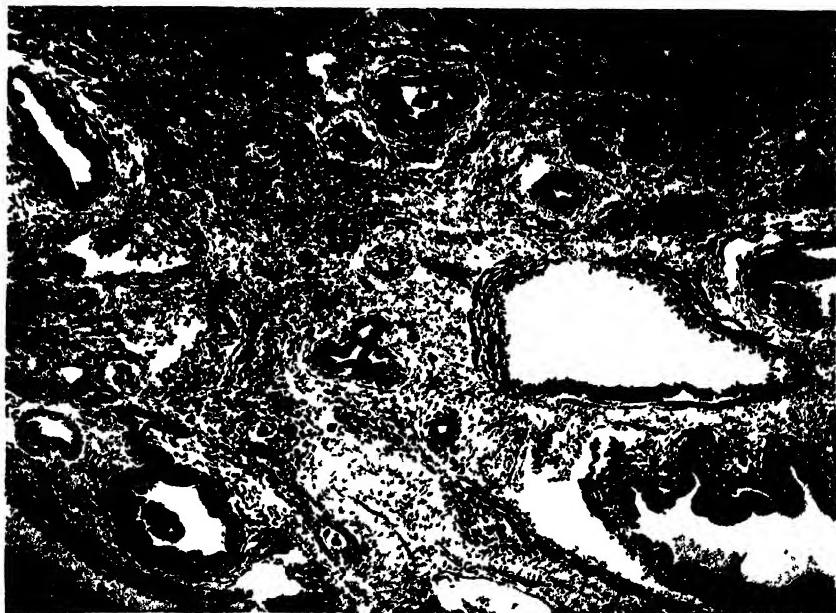


FIG. 10.

(Rous and Larimore Portal blood and liver maintenance )



## CRYSTALLINE URIDINPHOSPHORIC ACID.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 28, 1919.)

In previous communications<sup>1</sup> crystalline salts of uridinphosphoric acid were described. They were the brucine salt, two ammonium salts, the lead salt, and the barium salt. Nearly every one has its value in the process of preparation of the free uridinphosphoric acid. The insolubility of the brucine salt is the property on which is based the separation of this from a mixture containing other nucleotides. The ammonium salt is the transition step from the brucine salt to every other salt; the lead salt serves for separation of the nucleotide in its pure state, and hence for its identification when the nucleotide is available in only small quantities. For final identification the crystalline nucleotide offers the most satisfactory material. It was prepared in the following manner.

The diammonium salt described in the previous communication was converted into the lead salt. This was suspended in water, and through the suspension a stream of hydrogen sulfide gas was passed. The filtrate from lead sulfide was freed from hydrogen sulfide by distillation under diminished pressure at room temperature. To the clear solution of the nucleotide a solution of neutral lead acetate was added to form again the lead salt. The lead salt was again treated with hydrogen sulfide as before, the resulting clear solution was concentrated under diminished pressure to a small volume, and then placed in a vacuum desiccator, where it was allowed to concentrate slowly under diminished pressure. When the solution attained the thickness of glycerol it was dissolved in hot 99.5 per cent alcohol and again placed in a vacuum desiccator and allowed to concentrate under diminished pressure over sulfuric acid. This

<sup>1</sup> Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21; *J. Biol. Chem.*, 1918, xxxiii, 229; 1919, xl, 395.

operation was repeated many times and finally the thick syrup crystallized into a nearly solid, sticky mass. In order to separate the crystals from the viscous mother liquor the material was triturated with a very small quantity of hot anhydrous methyl alcohol. The crystalline material was then washed with cold methyl alcohol, and finally the substance was suspended in dry methyl alcohol. The mixture was brought to a boil and then the crystals were filtered off. The mother liquors and the wash alcohol on standing under diminished pressure over sulfuric acid gave a second crop of crystals. The substance had the melting point M.P. = 202° (corrected) with decomposition and the following composition.

0.1000 gm. of the substance gave 0.1231 gm. of CO<sub>2</sub> and 0.0376 gm. of H<sub>2</sub>O.  
 0.2000 " " " employed for Kjeldahl nitrogen estimation required 12.36 cc. of 0.1 N acid for neutralization.

0.3000 gm. of the substance gave 0.1020 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

	Calculated for C <sub>10</sub> H <sub>12</sub> N <sub>2</sub> PO <sub>4</sub> per cent	Found. per cent
C.....	33.32	33.40
H.....	4.05	4.18
N.....	8.64	8.65
P.....	9.58	9.48

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.21 \times 100}{1 \times 2} = +10.5$$

Thus every one of the four nucleotides composing the molecule of yeast nucleic acid has now been prepared in crystalline form.

## THE STRUCTURE OF YEAST NUCLEIC ACID.

### V. AMMONIA HYDROLYSIS.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 1, 1919.)

In the preceding publication of this series<sup>1</sup> the statement was made that on mild hydrolysis with 5 per cent ammonia at a temperature of 100°C. yeast nucleic acid is broken up into four mononucleotides. The publication contained a report of the isolation of only three of these. At the time when that publication went to press, the fourth nucleotide had been isolated in the form of the brucine salt. However, we have learned that for the identification of a nucleotide one cannot depend on the analysis of only the brucine salt, when this salt is obtained from a mixture of brucine salts of several nucleotides.

The present publication contains a report on the isolation of the fourth nucleotide, the crystalline cytidinphosphoric acid. It also presents an example showing that not only the brucine salts, but also the free nucleotides are capable of forming mixed crystals, which may furnish analytical data very nearly approaching those required by polynucleotides. A crystalline substance was isolated, which, on the basis of its elementary analysis, could easily have been taken for an adenosin-uridin dinucleotide. On recrystallization from dilute alcohol, the substance was fractionated into cytidinphosphoric and adenosinphosphoric acids. Thus it is evident that the danger of error is extremely great if one assumes the existence of di- or trinucleotides on the basis of the results of elementary analysis.

### EXPERIMENTAL.

The material for the present work was the brucine salts of the adenine fraction. It was stated in the preceding communication

<sup>1</sup> Levene, P. A., *J. Biol. Chem.*, 1919, xl, 415.

that these brucine salts were recrystallized nine times out of 35 per cent alcohol. The final product consisted of the brucine salt of uridinphosphoric in a practically pure state. This was demonstrated by the fact that, when the brucine salt was converted into the ammonium salt, this without further purification was in a crystalline state and had an elementary composition required by the theory of that salt.

The first three mother liquors combined and concentrated yielded a brucine salt containing 10 per cent nitrogen, which served for the isolation of adenosinphosphoric acid described in the preceding publication.

The mother liquors from the fourth to the ninth (inclusive) recrystallizations served as starting material for the isolation of cytidinphosphoric acid. The crude brucine salts obtained on concentration of the mother liquors were first fractionated by means of methyl alcohol. For this purpose the brucine salts were suspended in methyl alcohol, boiled for 15 minutes on boiling water bath, and filtered while hot. There were obtained an insoluble part A and the mother liquor, which on cooling deposited a sediment B; the mother liquor from B on concentration under diminished pressure gave a third precipitate C. The last was too small for further work. Fraction A was recrystallized three times out of 35 per cent alcohol. The brucine salt had a nitrogen content  $N = 7.71$  per cent. It was converted into the ammonium salt, and this into the lead salt. The latter was suspended in water. Through the suspension hydrogen sulfide gas was passed and the filtrate from lead sulfide was concentrated to small volume under diminished pressure, finally placed in vacuum desiccator over sulfuric acid, and allowed to crystallize under diminished pressure. When the solution was concentrated to the consistency of a syrup, there began to form a crystalline deposit consisting of long needles. The mother liquor was very viscous and the crystals were freed from it by repeated washing with hot methyl alcohol. Apparently the mother liquor contained some uridinphosphoric, which is extremely soluble in water.

The crystalline substance was analyzed without recrystallization. It had a melting point M. P. =  $225^{\circ}\text{C}$ . (corrected) with decomposition. The analysis of the substance was as follows.

0.1106 gm. of the substance employed for Kjeldahl nitrogen estimation required 10.34 cc. of 0.1 N acid.

	Calculated for $C_9H_{14}O_4N_2P$ per cent	Found. per cent
N.....	13.00	13.09

The optical rotation of the substance was as follows.

$$[\alpha]_D^{\infty} = \frac{+1.05 \times 100}{1 \times 2} = +52.5$$

The rotation of the cytidinphosphoric acid isolated by Thannhauser was  $[\alpha]_D^{\infty} = +23.3$ , and the value calculated from the barium salt described by the writer was also  $[\alpha]_D^{\infty} = +23$ . The reason for this discrepancy will have to be established. The two latter preparations were obtained on acid hydrolysis.

Fraction B was recrystallized four times out of 35 per cent alcohol. The resulting substance was converted into the ammonium salt, which in its turn was converted into the lead salt, and this was freed from lead and thus a solution of free nucleotides was obtained. This was concentrated under diminished pressure and the solution allowed to crystallize in the open air. Crystallization began rapidly and was completed after 3 days. The crystal form differed from that of the pure nucleotides. The latter appear in form of long fine needles, while these had the appearance of heavy prisms. The substance was recrystallized once out of hot water and then had the following composition.

0.1170 gm. of the substance gave 0.1453 gm. of  $CO_2$  and 0.0419 gm. of  $H_2O$ .

0.1930 gm. of the substance employed for Kjeldahl nitrogen estimation required 20.60 cc. of 0.1 N acid for neutralization.

0.2845 gm. of the substance gave 0.0954 gm. of  $Mg_2P_2O_7$ .

	Calculated for $C_{12}H_{20}O_11N_2P_2$ per cent	Found. per cent
C.....	34.90	33.86
H.....	3.86	4.12
N.....	15.01	14.94
P.....	9.50	9.19

The optical rotation of this substance was

$$[\alpha]_D^{\infty} = \frac{+0.40 \times 100}{1 \times 2} = +20.0$$

Thus the substance could easily be taken for a dinucleotide. It was separated into two fractions in the following manner: 4.8 gm. of the substance were dissolved in 150 cc. of hot water, and to this solution 300 cc. of 99.5 per cent alcohol were added gradually. On cooling a crystalline deposit began to form. After 1 hour this was removed by filtration. The yield of the air-dry material was 2.8 gm. (Fraction A). In the mother liquor on concentration under diminished pressure a deposit formed consisting of long needles (Fraction B).

Fraction A<sub>1</sub> analyzed as follows.

0.1006 gm. of the substance gave 0.1216 gm. of CO<sub>2</sub> and 0.040 gm. of H<sub>2</sub>O. 0.2000 " " " employed for Kjeldahl nitrogen estimation required 18.92 cc. of 0.1 N acid.

0.2940 gm. of the substance gave 0.0982 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.80 \times 100}{1 \times 2} = +40.0$$

Once recrystallized out of water the substance analyzed as follows.

0.0986 gm. of the substance gave 0.1206 gm. of CO<sub>2</sub> and 0.041 gm. of H<sub>2</sub>O.

0.1973 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 18.33 cc. of 0.1 N acid.

The optical rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.86 \times 100}{1 \times 2} = +43$$

	Calculated for C <sub>10</sub> H <sub>14</sub> O <sub>4</sub> N <sub>2</sub> P <sub>2</sub>	Found.	
	per cent	Sample A <sub>1</sub> . per cent	Sample A <sub>2</sub> . per cent
C.....	33.42	33.57	33.35
H.....	4.37	4.44	4.64
N.....	13.00	13.51	13.01
P.....	9.61	9.30	

Fraction B<sub>1</sub> analyzed as follows.

0.2000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 26.24 cc. of 0.1 N acid.

	Calculated for C <sub>12</sub> H <sub>14</sub> O <sub>7</sub> N <sub>2</sub> P. <i>per cent</i>	Found- <i>per cent</i>
N.....	20.17	18.44

The optical rotation of the substance was

$$[\alpha]_D^{\infty} = \frac{-0.78 \times 100}{1 \times 2} = -39.0$$



[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, April, 1920, Vol. xli, No. 4,  
pp. 483-493.]

## PROPERTIES OF THE NUCLEOTIDES OBTAINED FROM YEAST NUCLEIC ACID.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 4 AND 5.

(Received for publication, March 1, 1920.)

The four nucleotides composing the molecule of yeast nucleic acid have now been isolated in crystalline form. Of these adenosinphosphoric acid was isolated by different methods by Jones and Kennedy<sup>1</sup> and by Levene.<sup>2</sup> The two substances seemed to differ from one another on the point of the crystal water.

Cytidinphosphoric acid also was obtained in two laboratories (Thannhauser and Dorfmüller<sup>3</sup> and Levene<sup>4</sup>) and the two substances seemed to differ in their optical activity. Also the two samples isolated by the present writer showed minor differences in their rotatory power. The properties of guanosinphosphoric<sup>5</sup> and uridinphosphoric acids<sup>6</sup> had been given as found on substances as they were first obtained without further recrystallization.

The present work was undertaken for the sake of clearing up the above mentioned discrepancies and, further, for the sake of establishing the composition of the nucleotide as regards the crystal water, and for the sake of establishing the physical constants with greater rigor. In order to make the work possible, larger quantities of the crude material were required and were prepared. The results of the analysis of the carefully purified substance are as follows:

*Adenosinphosphoric acid* (Fig. 1), according to Jones and Kennedy,<sup>1</sup> crystallizes with 1 molecule of crystal water. Levene, in his original

<sup>1</sup> Jones, W., and Kennedy, R. P., *J. Pharm. and Exp. Therap.*, 1919, xiii, 45.

<sup>2</sup> Levene, P. A., *J. Biol. Chem.*, 1919, xl, 415.

<sup>3</sup> Thannhauser, S. J., and Dorfmüller, G., *Z. physiol. Chem.*, 1919, civ, 65.

<sup>4</sup> Levene, P. A., *J. Biol. Chem.*, 1920, xli, 19.

<sup>5</sup> Levene, P. A., *J. Biol. Chem.*, 1919, xl, 171.

<sup>6</sup> Levene, P. A., *J. Biol. Chem.*, 1920, xli, 1.

work, found the air-dry substance anhydrous. The observation could not be repeated. Many samples have been analyzed since the first publication and all contained 1 molecule of crystal water. This was easily removed by drying under diminished pressure at the temperature of xylene vapor for 24 hours. The original sample was reanalyzed and again found practically anhydrous (1 per cent of water). In a sealed capillary tube the air-dry substance decomposed with effervescence at 195°C. (corrected).

*Guanosinphosphoric acid* (Fig. 2) crystallized in long needles of the appearance of the crystals of guanosin. It crystallized with 2 molecules of crystal water, which could be removed completely on drying under diminished pressure at the temperature of xylene vapor. In a sealed capillary tube the air-dry substance softened and became semitransparent at 175°C. and melted at 180°C. (corrected).

*Uridinphosphoric acid* (Fig. 3) was recrystallized out of methyl alcohol. The crystals formed slowly on evaporation of the alcohol. The larger crystals were often superimposed by deposits of smaller crystals, hence the photographic reproduction of the crystals was difficult. Besides, the yield of the material was rather small. For this reason it was decided to measure the optical rotation also of the monoammonium salt, which has good physical properties and a convenient solubility. The free acid crystallized in elongated prisms with pointed ends and melted in a sealed capillary tube at 198.5°C.

*The monoammonium salt* crystallized in prismatic needles which contained no crystal water. The air-dry substance heated in a sealed tube contracted and turned semitransparent at 200°C. (corrected), and decomposed at 242°C.

*Cytidinphosphoric acid* (Fig. 4) on repeated recrystallizations appeared in form of elongated plates which contained no crystal water. In a sealed capillary tube the air-dry substance decomposed with effervescence at 230–233°C.

#### *Rotations.*

The rotations were measured: (a) in aqueous solution, (b) in a solution of 10 per cent hydrochloric acid, (c) in a 5 per cent aqueous solution of ammonia, (d) in a 2 per cent aqueous solution of caustic soda, and (e) in a 5 per cent solution of the same.

*Guanosin- and adenosinphosphoric acids* showed a minimum levo-rotation in hydrochloric acid solution, which increased in water and in aqueous ammonia, and reached a maximum in an aqueous solution of sodium hydroxide.

*Cytidinphosphoric acid* showed a maximum dextrorotation in aqueous solution, successively decreasing in aqueous ammonia, hydrochloric acid, 2 per cent sodium hydroxide, and turned levorotatory in 10 per cent sodium hydroxide.

*Uridinphosphoric acid* and its ammonium salt changed their optical rotation in the same direction as the preceding substance.

The changes in the optical rotation probably are the resultants of more than one factor. Among these the tautomeric changes in the basic radical of the substance possibly play an important part. Indeed, adenosin and uridin showed the same character of optical rotation as the corresponding nucleotides. On the other hand, the molecular rotation of the nucleoside and of the nucleotide are not identical.

## EXPERIMENTAL.

*Guanosinphosphoric Acid.*—10 gm. of the crystalline material (No. 26) described in a previous communication<sup>6</sup> were recrystallized out of water. One part of the substance was suspended in thirty parts of water and the water kept boiling until solution was completed. The solution was allowed to stand at room temperature (about 25°C.) over night. The yield of recrystallized material was 7.5 gm. (No. 291). It was planned to continue recrystallization until a constant optical rotation of the substance was attained. On the first recrystallization the specific rotation remained without change. No. 291 was recrystallized once more out of 200 cc. of water in the same manner as No. 26. The resulting material showed no change in its optical rotation. The air-dry substance heated in a sealed capillary tube contracted and turned semitransparent at 175°C. (corrected) and decomposed at 180°C.

0.1168 gm. of the air-dry substance on drying to constant weight under diminished pressure at the temperature of xylene vapor lost 0.1112 gm. in weight.

	Calculated for $C_{16}H_{14}NiPO_4 + 2H_2O$	Found.
	per cent	per cent
$H_2O$ .....	9.47	9.67

The dry substance (No. 312) analyzed as follows:

0.1056 gm. of the substance gave 0.1312 gm. of CO<sub>2</sub> and 0.0168 gm. of H<sub>2</sub>O.  
 0.1807 gm. of the substance employed for Kjeldahl nitrogen estimation required  
 for neutralization 34.85 cc. of 0.1 N acid.  
 0.2710 gm. of the substance gave 0.0839 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

	Calculated for C <sub>10</sub> H <sub>14</sub> N <sub>2</sub> PO <sub>4</sub> per cent	Found. per cent
C.....	33.05	33.89
H.....	3.89	3.90
N.....	19.22	19.25
P.....	8.55	8.62

Rotation of the substance in aqueous solution:

$$\text{No. 26} \quad [\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5^\circ$$

$$\text{No. 291} \quad [\alpha]_D^{25} = \frac{-0.15 \times 100}{1 \times 2} = -7.5^\circ$$

$$\text{No. 312} \quad [\alpha]_D^{25} = \frac{-0.16 \times 100}{1 \times 2} = -8.0^\circ$$

Rotation of the air-dry substance in 10 per cent hydrochloric acid:

$$\text{No. 312} \quad [\alpha]_D^{25} = \frac{+0.03 \times 100}{1 \times 2} = +1.5^\circ$$

Rotation of the air-dry substance in 5 per cent aqueous ammonia solution:

$$\text{No. 312} \quad [\alpha]_D^{25} = \frac{-0.88 \times 100}{1 \times 2} = -44.0^\circ$$

The rotation of the substance in 2 per cent aqueous caustic soda was as follows:

$$[\alpha]_D^{25} = \frac{-1.14 \times 100}{1 \times 2} = -57.0^\circ$$

In 5 per cent of the same:

$$[\alpha]_D^{25} = \frac{-1.30 \times 100}{1 \times 2} = -65.0^\circ$$

*Adenosinphosphoric acid* is the most insoluble of the four nucleotides. 23 gm. of the substance (No. 286) were suspended in 1 liter of hot water and the water was kept boiling for some time and since the substance did not dissolve readily dilute ammonia was added gradually until solution was completed. The hot solution was rendered slightly acid to litmus by means of acetic acid and allowed to stand at room temperature over night. A crystalline deposit (No. 282) formed.

The precipitate thus formed was recrystallized out of 600 cc. of water and allowed to crystallize over night. A crystalline deposit was formed. The yield was 7.5 gm. (No. 290). These were again recrystallized out of hot water. The yield was 5 gm. (No. 306). On heating in a sealed capillary tube the substance decomposed at 195°C. (corrected).

#### Analysis of the substance:

0.1325 gm. of the substance on drying under diminished pressure at the temperature of xylene vapor lost 0.0069 gm. of water.

	Calculated for $C_{10}H_{14}N_6PO_7 + H_2O$ .	Found.
	per cent	per cent
$H_2O \dots \dots \dots$	4.90	5.20

Several samples were analyzed with the same result whereas the first two samples were analyzed for the anhydrous substance.

The dry substance (No. 306) analyzed as follows:

0.1256 gm. of the substance gave on combustion 0.1599 gm. of  $CO_2$  and 0.0456 gm. of  $H_2O$ .

0.0948 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 13.56 cc. of 0.1 N acid.

0.2844 gm. of the substance gave 0.0913 gm. of  $Mg_2P_2O_7$ .

	Calculated for $C_{10}H_{14}N_6PO_7$ .	Found.
	per cent	per cent
C. ....	34.57	34.71
H. ....	4.07	4.06
N. ....	20.17	20.13
P. ....	8.94	9.04

Rotation of the substance in 1 per cent aqueous solution in 2 dm. tube:

$$\text{No. 306} \quad [α]_D^{\text{w}} = \frac{-0.81 \times 100}{2 \times 1} = -40.5^\circ$$

Since the substance is only little soluble in water the readings of the substances in process of their purification were taken in 5 per cent aqueous ammonia.

Rotation in 5 per cent aqueous ammonia solution:

$$\text{No. 282} \quad [\alpha]_D^{20} = \frac{-0.81 \times 100}{1 \times 2} = -40.5^\circ$$

$$\text{No. 290} \quad [\alpha]_D^{20} = \frac{-0.89 \times 100}{1 \times 2} = -44.5^\circ$$

$$\text{No. 306} \quad [\alpha]_D^{20} = \frac{-0.83 \times 100}{1 \times 2} = -41.5^\circ$$

The slight variation might be due to variation in the moisture of the substance.

Rotation in a solution of 10 per cent hydrochloric acid:

$$\text{No. 306} \quad [\alpha]_D^{20} = \frac{-0.76 \times 100}{1 \times 2} = -38.00^\circ$$

The rotation of the substance in 2 per cent aqueous caustic soda was as follows:

$$[\alpha]_D^{20} = \frac{-1.19 \times 100}{1 \times 2} = -59.5^\circ$$

In 5 per cent of the same:

$$[\alpha]_D^{20} = \frac{-1.32 \times 100}{1 \times 2} = -66.0^\circ$$

*Cytidinphosphoric Acid.*—The starting material was a mixture of cytidin- and adenosinphosphoric acid, prepared in the manner described in the previous paper. The original optical rotation was  $[\alpha]^\infty = +25$ . By recrystallization out of 50 per cent alcohol finally a substance was obtained with  $[\alpha]_D^{20} = +40$ . 10.0 gm. of this material (No. 270) were dissolved in 500 cc. of boiling water and to the solution 500.0 cc. of 99.8 per cent alcohol were added. Soon heavy crystals began to settle out and the crystallization was allowed to proceed 48 hours. The yield of the crystals was 7.5 gm. (No. 280). This material was then dissolved in 400 cc. of boiling water and 400 cc. of 99.8 per cent alcohol were added to the solution. The crystalliza-

tion proceeded as above. The yield of the crystals was 6.0 gm. (No. 285). These 6.0 gm. were dissolved in 350 cc. of boiling water and to the solution 150.0 cc. of 99.8 per cent alcohol were added. The yield of the final material (No. 289) was 5.0 gm. The crystal form is reproduced in Fig. 4.

The substance in sealed capillary tube decomposed with effervescence at 230–233°C. (corrected). (Heating slow.)

The analysis of the substance was as follows:

On drying under diminished pressure at the temperature of xylene vapor the substance lost 0.8 per cent in weight.

0.1123 gm. of the dry substance gave 0.1374 gm. of CO<sub>2</sub> and 0.0442 gm. of H<sub>2</sub>O. 0.0992 gm. of the dry substance employed for Kjeldahl nitrogen estimation required for neutralization 9.23 cc. of 0.1 N acid.

0.2976 gm. of the substance gave 0.1014 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

	Calculated for C <sub>9</sub> H <sub>14</sub> N <sub>2</sub> PO <sub>8</sub> . per cent	Found. per cent
C.....	33.42	33.36
H.....	4.37	4.41
N.....	13.00	13.03
P.....	9.61	9.50

The optical rotation was as follows:

In aqueous solution:

$$\text{No. 270} \quad [\alpha]_D^{20} = \frac{+ 0.80 \times 100}{1 \times 2} = + 40.0^\circ$$

$$\text{No. 285} \quad [\alpha]_D^{20} = \frac{+ 0.97 \times 100}{1 \times 2} = + 48.5^\circ$$

$$\text{No. 289} \quad [\alpha]_D^{20} = \frac{+ 0.95 \times 100}{1 \times 2} = + 47.5^\circ$$

In 10 per cent hydrochloric acid solution:

$$\text{No. 289} \quad [\alpha]_D^{20} = \frac{+ 0.52 \times 100}{1 \times 2} = + 26.0^\circ$$

In 5 per cent ammoniacal solution:

$$\text{No. 289} \quad [\alpha]_D^{20} = \frac{+ 0.89 \times 100}{1 \times 2} = + 44.5^\circ$$

In 2 per cent sodium hydroxide solution:

$$\text{No. 289} \quad [\alpha]_D^{\infty} = \frac{+ 0.51 \times 100}{1 \times 2} = + 25.5^\circ$$

In 5 per cent solution of the same:

$$[\alpha]_D^{\infty} = \frac{+ 0.02 \times 100}{1 \times 2} = + 1.0^\circ$$

In 10 per cent solution of the same:

$$[\alpha]_D^{\infty} = \frac{- 0.42 \times 100}{1 \times 2} = - 21.0^\circ$$

*Uridinphosphoric Acid.*—The material described in a previous communication was dissolved in boiling methyl alcohol and the solution was allowed to stand in a desiccator over sulfuric acid. On standing, after a considerable part of the alcohol evaporated, heavy crystals settled out on the walls of the dish. The crystallization proceeded very slowly.

The substance melted in sealed tube with decomposition at M.P. = 198.5°C. (corrected).

The analysis of the substance was as follows:

0.1000 gm. of the substance used for Kjeldahl nitrogen estimation required for neutralization 5.97 cc. of 0.1 N acid.

	Calculated for $C_9H_{12}N_2PO_6$ per cent	Found. per cent
N.....	8.64	8.36

The rotation of the substance (No. 343) was as follows:

In aqueous solution:

$$[\alpha]_D^{\infty} = \frac{+ 0.19 \times 100}{1 \times 2} = + 9.5^\circ$$

In 2 per cent solution of sodium hydroxide:

$$[\alpha]_D^{\infty} = \frac{+ 0.13 \times 100}{1 \times 2} = + 6.5^\circ$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{\infty} = \frac{- 0.30 \times 100}{1 \times 2} = - 15.0^\circ$$

*Monoammonium Salt of Uridinphosphoric Acid.*—The crystalline material described in a previous communication was dissolved in a minimum amount of water and to the solution 20 volumes of methyl alcohol were added. The solution was allowed to stand at room temperature. After several days on walls of the flask a sediment formed, which consisted of fine curved felt-forming needles (No. 254). In the mother liquor, on further standing, a third precipitate formed. The crystal form is reproduced in Fig. 3.

The substance when heated in a sealed capillary tube contracted and turned semitransparent at 200°C. (corrected) and decomposed with effervescence at 240°C.

The analysis of the substance was as follows:

0.1204 gm. of the substance gave on combustion 0.1417 gm. of CO<sub>2</sub> and 0.0514 gm. of H<sub>2</sub>O.

0.1964 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 17.24 cc. of 0.1 N acid.

0.1964 gm. of the substance gave 0.0650 gm. of Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

	Calculated for C <sub>4</sub> H <sub>11</sub> N <sub>2</sub> P <sub>2</sub> O <sub>9</sub> , per cent	Found, per cent
C.....	31.66	32.09
H.....	4.73	4.77
N.....	12.32	12.30
P .....	9.58	9.15

The optical rotation of the substance was as follows:

In aqueous solution:

$$\text{No. 255} \quad [\alpha]_D^{25} = \frac{+ 0.21 \times 100}{1 \times 2} = + 10.5^\circ$$

In 10 per cent hydrochloric acid solution:

$$\text{No. 255} \quad [\alpha]_D^{25} = \frac{+ 0.05 \times 100}{1 \times 2} = + 2.5^\circ$$

In 5 per cent ammoniacal solution:

$$\text{No. 255} \quad [\alpha]_D^{25} = \frac{+ 0.28 \times 100}{1 \times 2} = + 14.0^\circ$$

The rotation of the substance in 2 per cent aqueous sodium hydroxide was:

$$[\alpha]_D^{20} = \frac{+ 0.03 \times 100}{1 \times 2} = + 1.5^\circ$$

In 5 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{- 0.32 \times 100}{1 \times 2} = - 16.0^\circ$$

In 10 per cent solution of the same:

$$[\alpha]_D^{20} = \frac{- 0.52 \times 100}{1 \times 2} = - 26.0^\circ$$

The optical rotation of adenosin was as follows:

In aqueous solution:

$$[\alpha]_D^{20} = \frac{- 1.20 \times 100}{1 \times 2} = - 60.0^\circ$$

In 10 per cent solution of hydrochloric acid:

$$[\alpha]_D^{20} = \frac{- 0.87 \times 100}{1 \times 2} = - 43.5^\circ$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{- 1.37 \times 100}{1 \times 2} = - 68.5^\circ$$

The optical rotation of uridin was as follows:

In aqueous solution:

$$[\alpha]_D^{20} = \frac{+ 0.08 \times 100}{1 \times 2} = + 4.0^\circ$$

In 10 per cent solution of hydrochloric acid:

$$[\alpha]_D^{20} = \frac{+ 0.10 \times 100}{1 \times 2} = + 5.0^\circ$$

In 5 per cent solution of sodium hydroxide:

$$[\alpha]_D^{20} = \frac{- 0.12 \times 100}{1 \times 2} = - 6.0^\circ$$

EXPLANATION OF PLATES.

PLATE 4.

- FIG. 1. Crystals of adenosinphosphoric acid.  
FIG. 2. Crystals of guanosinphosphoric acid.

PLATE 5.

- FIG. 3. Crystals of monoammonium salt of uridinphosphoric acid.  
FIG. 4. Crystals of cytidinphosphoric acid.



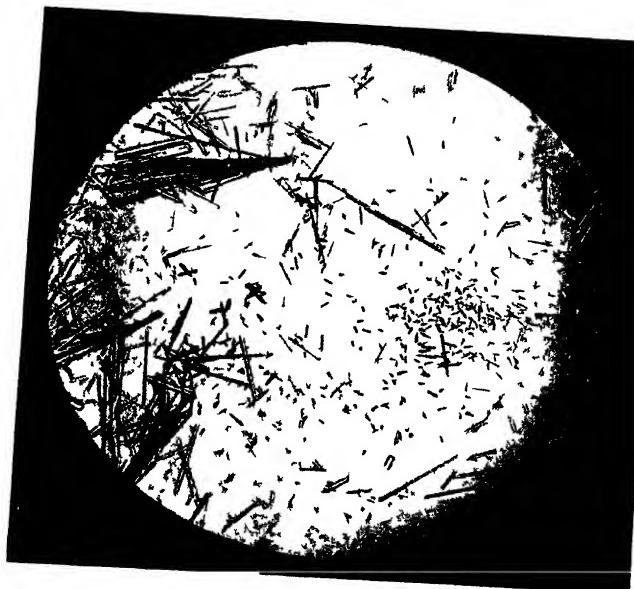


FIG. 2.

(Levene Nucleotides from yeast nucleic acid)





FIG. 3.

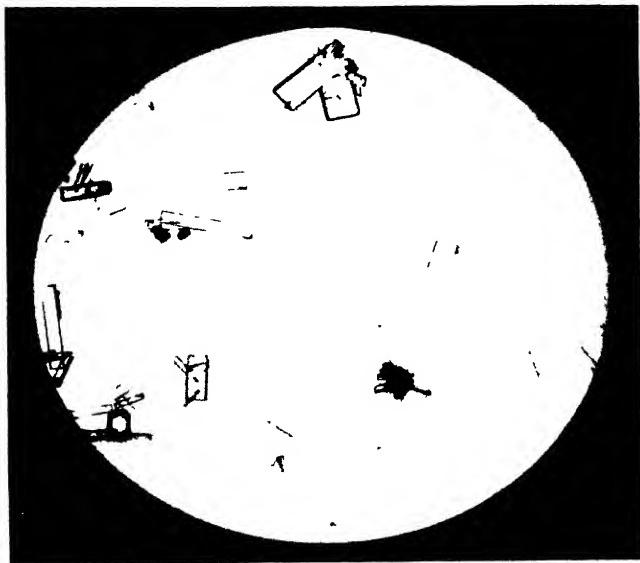


FIG. 4.

(Levene: Nucleotides from yeast nucleic acid.)



[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, January 20, 1920, Vol. ii,  
No. 3, pp. 255-271.]

## INFLUENCE OF A SLIGHT MODIFICATION OF THE COLLODION MEMBRANE ON THE SIGN OF THE ELECTRIFICATION OF WATER.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 25, 1919.)

### I.

When we separate a solution of an electrolyte from pure water by a collodion bag the water will as a rule diffuse into the solution. In a preceding paper<sup>1</sup> it was shown that the forces determining this diffusion are different for concentrations of electrolytes below or above a certain value (which is for certain sodium salts about  $m/16$ ). The forces causing the diffusion of water into the solution below this critical concentration are predominantly electrical, while the forces causing the diffusion above the critical concentration are predominantly (or perhaps exclusively) molecular.

The electrical forces of diffusion depend upon the sign, the valency, a third property of the ions (which we arbitrarily designated as their radius), and in addition upon the concentration of the ions in solution. There is still another variable to be considered; namely, the nature of the membrane. We have already called attention to the fact that a collodion membrane which has once been treated with a gelatin solution shows a different osmotic behavior from a membrane not treated with gelatin. The gelatin treatment consisted in this that the collodion bags with which the experiments were made were filled over night with a 1 per cent gelatin solution (isoelectric or nearly isoelectric). The next day the gelatin solution was poured off and the interior of the bags washed out about six times or more with warm water to remove as much gelatin as possible. They were then put for days into water to dissolve still more and remove the last traces of gelatin.

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173.

Such bags seemed to retain the effect of the gelatin treatment for a long time, and continued use and subsequent washing did not seem to remove this after effect of the gelatin treatment.

When collodion bags which had received this gelatin treatment were filled with solutions of electrolytes (of the theoretical osmotic pressure of that of a M/64 sugar solution) and were dipped into beakers with distilled water, the influence of the nature of the electrolyte upon the initial rate of diffusion of water into the bag could be expressed by the following two rules.<sup>2</sup>

1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion. The same rule applies to solutions of alkalies.

2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion. Solutions of acids obey the same rule.

When the same experiments were repeated with collodion bags which had not come in contact with gelatin, the influence of the electrolytes mentioned in Rule 1 on the diffusion of water was the same as when the membrane had been treated with gelatin. Rule 2, however, was not valid when the collodion membranes had *not* been treated with gelatin. It was of interest to discover the cause of this difference.

The curves in Figs. 1 and 2 show that Rule 1 holds also for collodion membranes *not* treated with gelatin. The solutions of salts used were neutral or slightly alkaline (in the case of Na<sub>3</sub> citrate). The abscissæ in the figures are the logarithms of the concentration, the ordinates the height to which the level of liquid in the manometer rose in the first 20 minutes. The curves in Fig. 1 show that the levels

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 717.

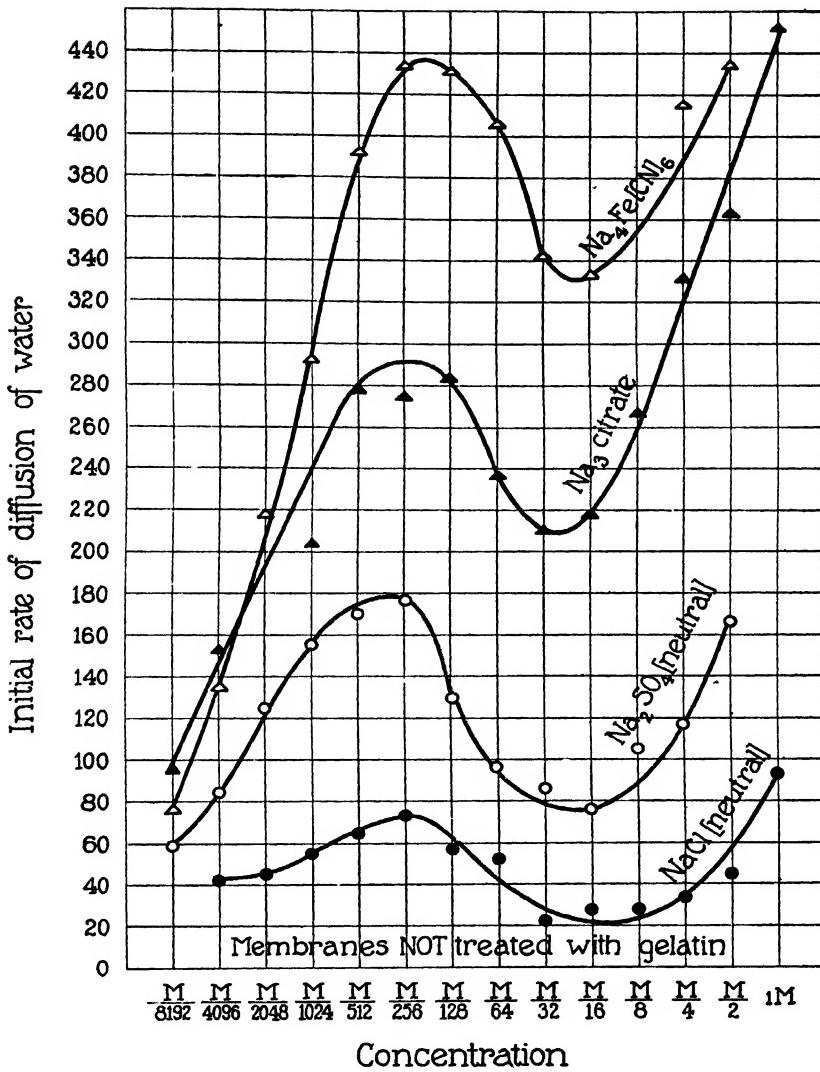


FIG. 1. Curves representing influence of concentration of solutions of different Na salts upon the initial rate of diffusion of water from pure water into solution through membranes not treated with gelatin. The curves are essentially the same as those obtained with collodion membranes which had been treated with gelatin.\* Abscissæ are the logarithms of concentration, ordinates the rise of height of level of solution in 20 minutes. Inside of collodion bag salt solution, outside  $\text{H}_2\text{O}$ .

\* Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 177, Fig. 3.

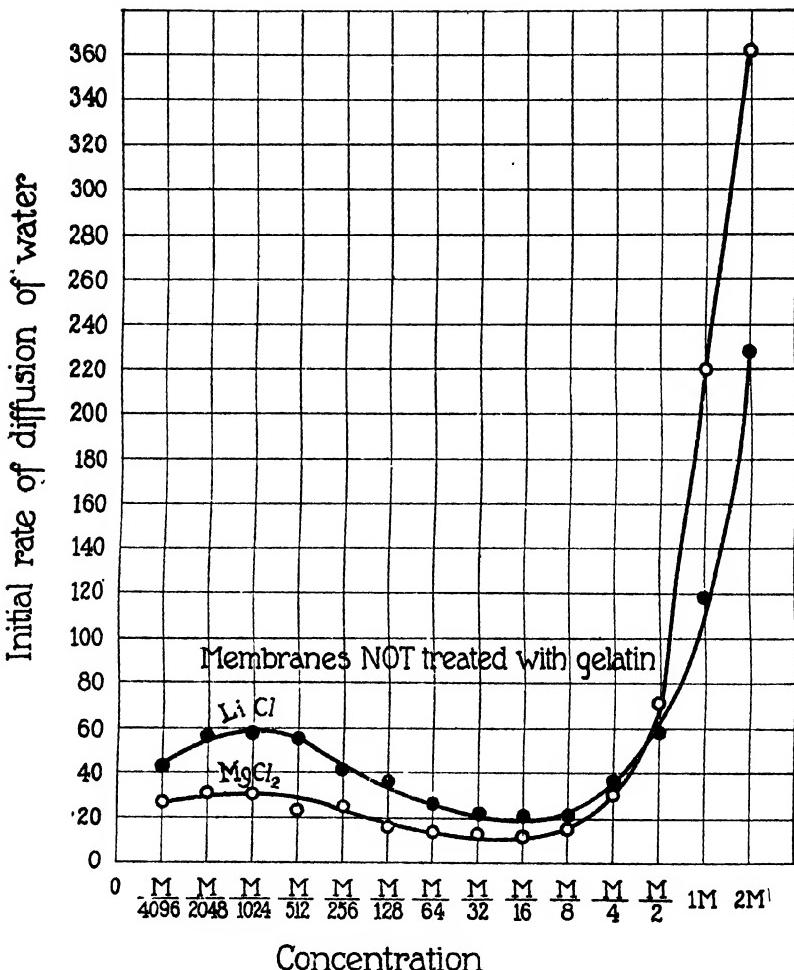


FIG 2. Same curves as in Fig. 1 for LiCl and MgCl<sub>2</sub>. Curves identical for membranes treated and not treated with gelatin.

at first rise sharply with an increase in concentration and the more so the higher the valency of the anion. The maximum of the curves is reached at about  $M/256$ , then the curves fall with a further increase in the concentration until a minimum is reached again at about  $M/16$ , and then another rise begins. This second rise expresses the gas pressure effect of the solute. When we use membranes treated with gelatin, we get a similar system of curves for the same solutions,<sup>1</sup> and the explanation of the curves is the same for both kinds of membrane. The particles of water diffuse through the membrane as if they were positively charged, being attracted by the anion of the salt and repelled by the cation, the attraction increasing with the valency of the anion. That the repulsion increases with the valency of the cation is shown in Fig. 2 where the lower curve represents the rate of diffusion of water in 20 minutes into  $MgCl_2$  solutions and the upper curve the initial rate of diffusion of water in 20 minutes into solutions of  $LiCl$  through membranes not treated with gelatin. These curves are also practically identical with those obtained for the same salt solutions when the collodion membrane had previously been treated with gelatin.<sup>1</sup> Hence Rule 1 holds in all essentials equally for membranes treated and not treated with gelatin.

The situation is altogether different for solutions of those electrolytes whose influence is described in Rule 2; namely, acids and neutral or acid solutions of salts with trivalent or tetravalent cation.

When we separate solutions of different concentrations of  $Al_2Cl_6$  from  $H_2O$  by collodion bags treated with gelatin, water diffuses very rapidly into the solution and the level of liquid in the manometer rises steeply with an increase in concentration, as is shown by the upper curve in Fig. 3. Water is negatively charged and is powerfully attracted by the trivalent cation Al. When we repeat the same experiment with membranes *not* treated with gelatin (lower curve of Fig. 3), we notice that in that range of concentrations of  $Al_2Cl_6$  where the diffusion is determined chiefly (or exclusively) by electrical forces no rise occurs until the concentration of the solution of  $Al_2Cl_6$  is about  $M/64$ ; at about this concentration the gas pressure effect of solutions of cane sugar begins to be noticeable. It, therefore, looks as if solutions of  $Al_2Cl_6$  showed no electrical but only the gas pressure effect when separated from pure water by a membrane not treated with

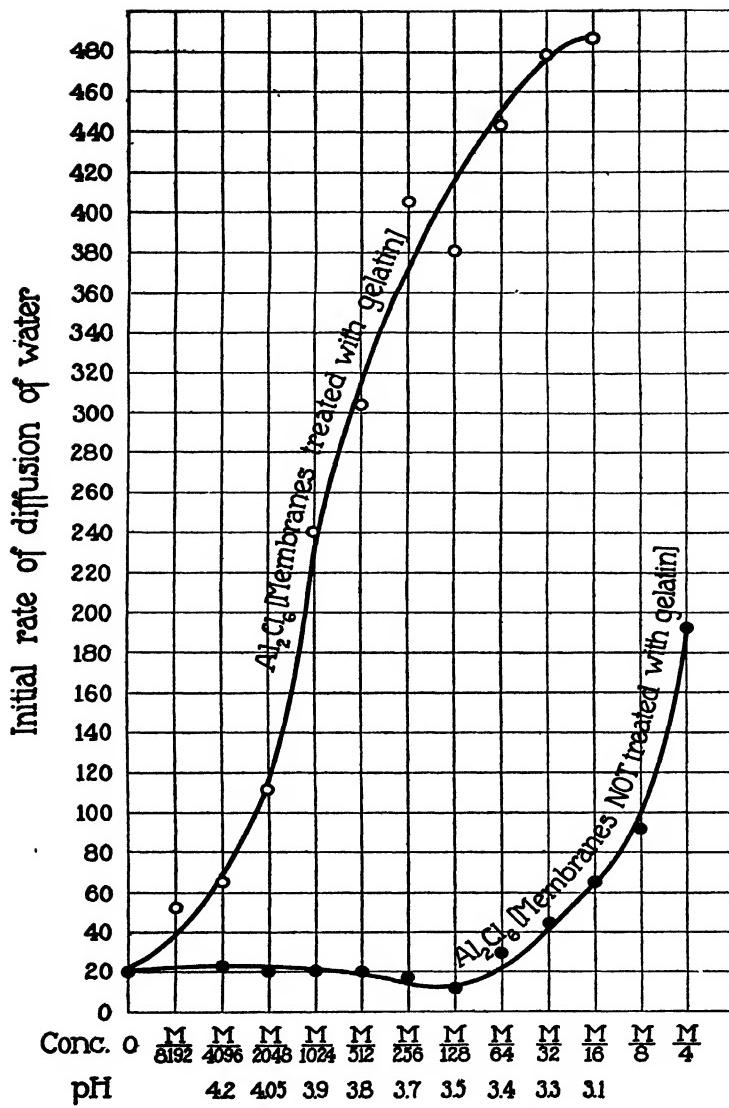


FIG. 3. Showing difference in osmotic behavior of collodion membranes treated and not treated with gelatin. Membranes separating solutions of  $\text{Al}_2\text{Cl}_6$  from pure water. Upper curve, initial rate of diffusion of water (first 20 minutes) into solution through membranes treated with gelatin; lower curve, diffusion of water during the first 20 minutes into same solution of  $\text{Al}_2\text{Cl}_6$  through membranes *not* treated with gelatin. In second case only the gas pressure effect of solution is apparent, while electrical attraction of water by solution is lacking.

gelatin. We shall see later that the electrical effect in the lower range of concentrations of  $\text{Al}_2\text{Cl}_5$  is not lacking, but that the low ordinates are due to the action of the trivalent cation. Curves for the diffusion of water into solutions of  $\text{LaCl}_3$  (Fig. 4), which are nearly neutral, show the same difference as those just described for  $\text{Al}_2\text{Cl}_5$  solutions. When  $\text{LaCl}_3$  solutions are separated from water by membranes not treated with gelatin, the ordinates (lower curve in Fig. 4) are low in the region of the electrical effect, while they rise steeply in the same region when the membranes have been treated with gelatin (upper curve in Fig. 4).

The difference in the osmotic behavior of the two kinds of membranes is still more striking when we use solutions of acids. When we separate solutions of strong acids (*e.g.*  $\text{HCl}$ ,  $\text{HNO}_3$ ,  $\text{H}_2\text{SO}_4$ ) from distilled water by collodion membranes previously treated with gelatin, we notice no rise but only a drop (Fig. 5)—negative osmosis—which commences for  $\text{H}_2\text{SO}_4$  and  $\text{H}_3\text{PO}_4$  at a concentration of about  $\text{M}/256$ . In the experiments represented in Fig. 5, the pressure head of the solution of acids inside the bag was about 70 mm. at the beginning. It dropped in 20 minutes to about 10 mm. at concentrations of about  $\text{M}/8$  or  $\text{M}/4$  for  $\text{H}_2\text{SO}_4$  and  $\text{H}_3\text{PO}_4$  (Fig. 5). Owing to the fact that the drop is due to the repelling action of the anion of the acid upon the negatively charged particles of water, the drop in the curves is greater when the anion of the acid is bivalent or trivalent than when it is monovalent. When we separate various concentrations of the same acids from pure water by membranes *not* treated with gelatin we get results of an altogether different order (Fig. 6). Instead of negative osmosis we notice a powerful positive osmosis, *i.e.* a rapid diffusion of water into the solution, and the acids behave almost like the sodium salts with the same anion. By comparing Fig. 1 and Fig. 6 the reader will notice the same steep rise of the curves until the concentration is about  $\text{M}/256$ ; this rise is more considerable in the case of  $\text{Na}_2\text{SO}_4$  and  $\text{H}_2\text{SO}_4$  than in the case of  $\text{NaCl}$  and  $\text{HCl}$ . In the case of these two salts and acids, the rise is followed by a drop, until for the acids at  $\text{M}/32$  and for the salts at  $\text{M}/16$  the gas pressure effect of the solution commences. The drop is not noticeable in the case of  $\text{H}_4\text{PO}_4$ .

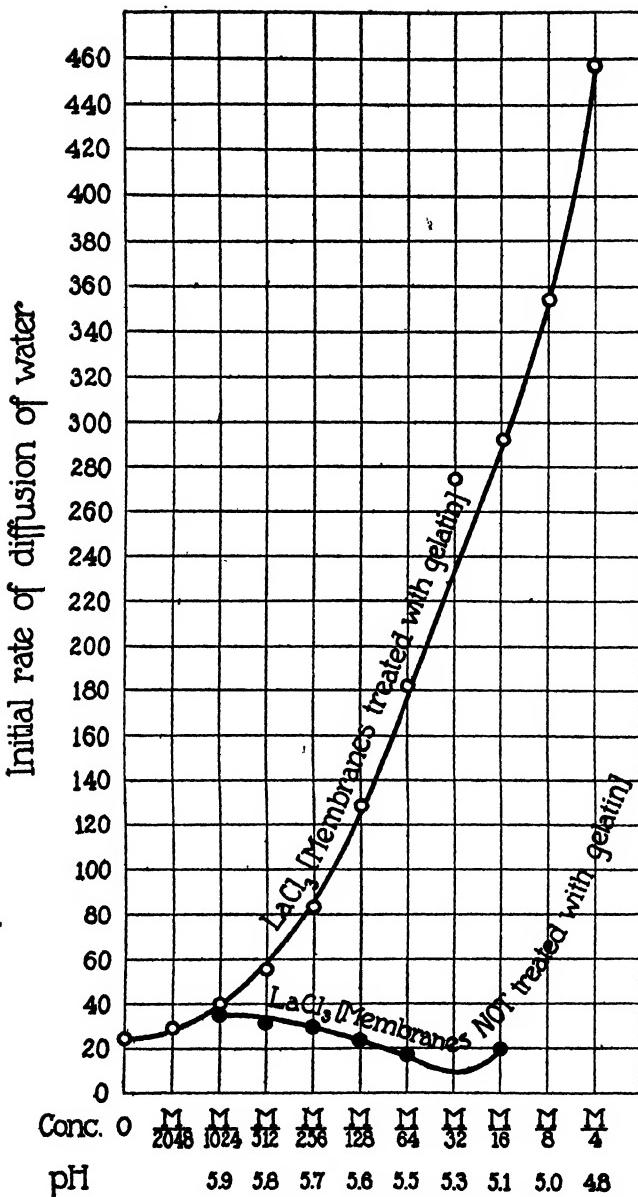


FIG. 4. Showing the same difference of membranes treated and not treated with gelatin for solutions of  $\text{LaCl}_3$ .

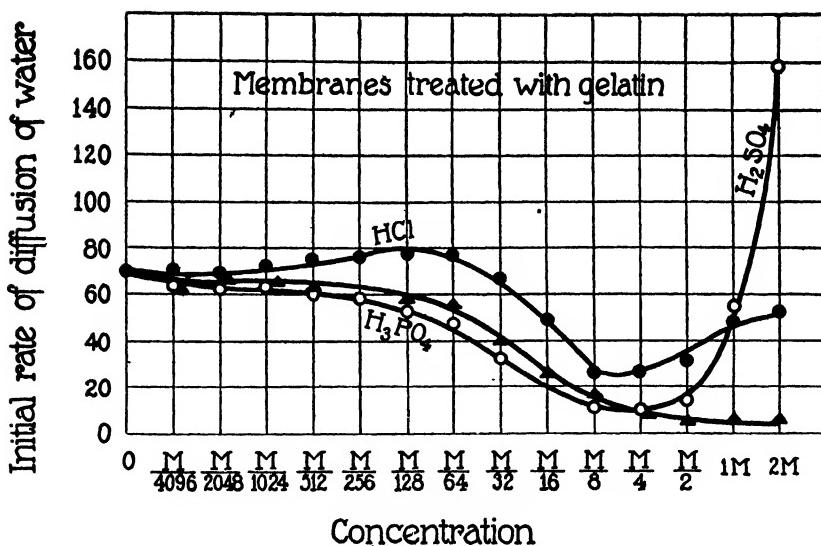


FIG. 5. Negative osmosis when acids ( $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{H}_3\text{PO}_4$ ) are separated from water by membranes treated with gelatin.

Fig. 7 gives the curves for several other acids when the membranes are treated with gelatin and Fig. 8 the curves for the same acids when the membranes are *not* treated with gelatin. When the membranes are *not* treated with gelatin the curve for oxalic acid (Fig. 8) becomes almost like that of  $\text{Na}_2\text{SO}_4$  in Fig. 1; while the same acid shows the phenomenon of negative osmosis (Fig. 7) when the membrane has received a gelatin treatment previous to the experiment.

## II.

What causes this profound difference in the osmotic behavior of collodion membranes according to whether they have or have *not* received a previous treatment with gelatin? It is so customary in biology to explain obscure phenomena by the assumption of variations of permeability that the writer felt it necessary to test the possibility of such an explanation in this case.

The phenomenon of positive osmosis for acid when the collodion bag separating the acid from pure water was not treated with gelatin, and the opposite phenomenon of negative osmosis when the collodion

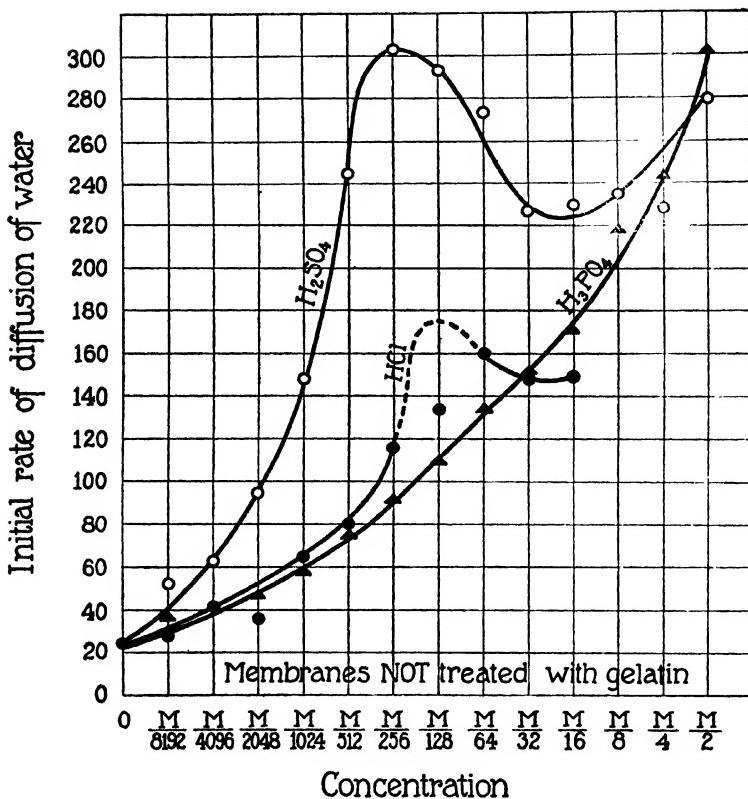


FIG. 6. Positive osmosis when the same acids as in Fig. 5 are separated from water by membranes *not* treated with gelatin. Curves similar to those obtained with sodium salts of the same anion (Fig. 1).

bag separating the acid had been treated with gelatin, suggested that the gelatin treatment had rendered the collodion bag more permeable for acid. It was, therefore, expected that titration of the contents of the bag at the beginning and after 20 minutes would show a greater diminution of the concentration of acid inside the bag when the bag had been treated with gelatin than when it had received no such treatment. The test, however, disproved the correctness of this explanation. *The rate of diffusion of acid from solution into the distilled water was practically the same for collodion membranes treated and not treated with gelatin*, as Table I shows.

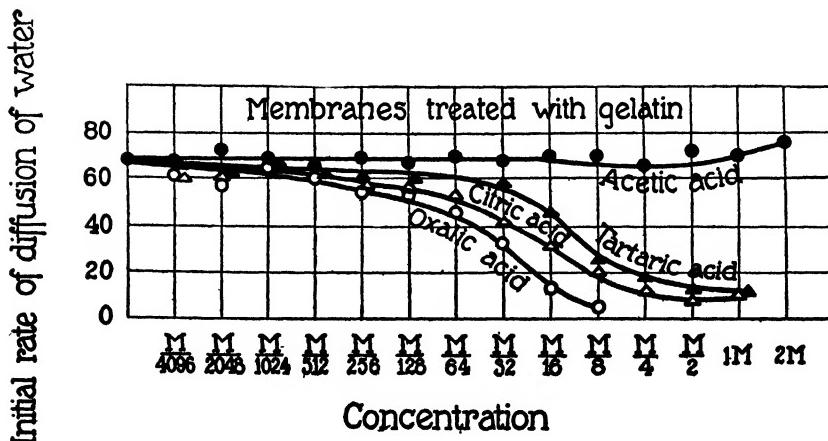


FIG. 7. Negative osmosis when solutions of acids (tartaric, citric, and oxalic) are separated from water by membranes treated with gelatin. The weak acetic acid behaves practically like a non-electrolyte.

The table shows that the amount of acid which diffused out from the collodion bag into the distilled water was (within the limits of accuracy of these experiments) the same for membranes treated and *not* treated with gelatin. Oxalic acid diffused a little more slowly than hydrochloric acid, but the rate of diffusion for each acid is approximately in direct proportion to its concentration. The fact that the same acids caused positive osmosis when the membranes were *not* treated with gelatin and negative osmosis when they were treated cannot be ascribed to differences in the permeability of the two types of collodion membranes.

Since solutions of  $\text{Al}_2\text{Cl}_6$  and of  $\text{LaCl}_3$  caused a vigorous diffusion of water from solvent into solution when the collodion membrane was treated with gelatin, but not when the membrane had *not* received a gelatin treatment, the idea suggested itself that the collodion membranes not treated with gelatin were so permeable for the molecules of the salt that an increase in volume of the solution by a diffusion of water into it was impossible. Titration experiments with  $\text{Al}_2\text{Br}_6$  proved, however, that there was only a slight difference in the rate.

TABLE I.

	Loss of cc. of 0.01 N acid for 10 cc. of solution in 20 min.		
	N/32	N/64	N/128
Membranes treated with gelatin.			
HCl.....	10.2	5.0	2.3
H <sub>2</sub> SO <sub>4</sub> .....	9.7	4.7	2.2
Oxalic acid.....	7.8	4.0	1.9
Membranes not treated with gelatin.			
HCl.....	11.0	4.9	2.3
H <sub>2</sub> SO <sub>4</sub> .....	9.8	4.1	1.7
Oxalic acid.....	8.1	3.4	1.6

of diffusion of Al<sub>2</sub>Br<sub>6</sub> through the two types of membranes; the non-treated membrane being slightly more permeable. The difference was, however, much too small to account for the difference in the osmotic behavior of the two types of membranes.

It was, therefore, necessary to look for another explanation in the different osmotic behavior of the two types of membranes. It seemed these differences would find their explanation if it could be proved that water diffuses through collodion membranes not treated with gelatin in the form of positively charged particles even in the presence of acids or salts with trivalent or tetravalent cation. For this purpose the method of electrical endosmose was applied. Identical solutions of LaCl<sub>3</sub> were put inside and outside a collodion bag not treated with gelatin and a current (of about 4.8 milliamperes and about 90 volts) was sent through the solution. The water migrated to the cathode, showing that its particles were positively charged in the presence of LaCl<sub>3</sub>. When the same experiment was repeated with membranes previously treated with gelatin it was found that water is negatively charged in the presence of solutions of salts with trivalent cation. It was furthermore possible to show that in the presence of acids water diffuses through membranes not previously treated with gelatin in the form of positively charged particles.

We can now understand why it is that water cannot diffuse through collodion membranes not treated with gelatin into solutions of salts with trivalent cation, *e.g.* AlCl<sub>3</sub> or LaCl<sub>3</sub> (in the region of electrical effect), since these particles of water carrying a positive charge are

repelled by the trivalent cation. We understand also why the initial rate of diffusion of water through membranes *not* treated with gelatin is greater into a solution of  $H_2SO_4$  than into a solution of  $HCl$ , since the positively charged particles of water are attracted by the anion of the acid and the more powerfully the higher the valency of the anion.

A very simple test will demonstrate that the water diffuses through collodion membranes *not* treated with gelatin in the form of positively charged particles even in the presence of  $M/1,000$  acid. We know from Rule 1 (at the beginning of this paper) that positively charged particles

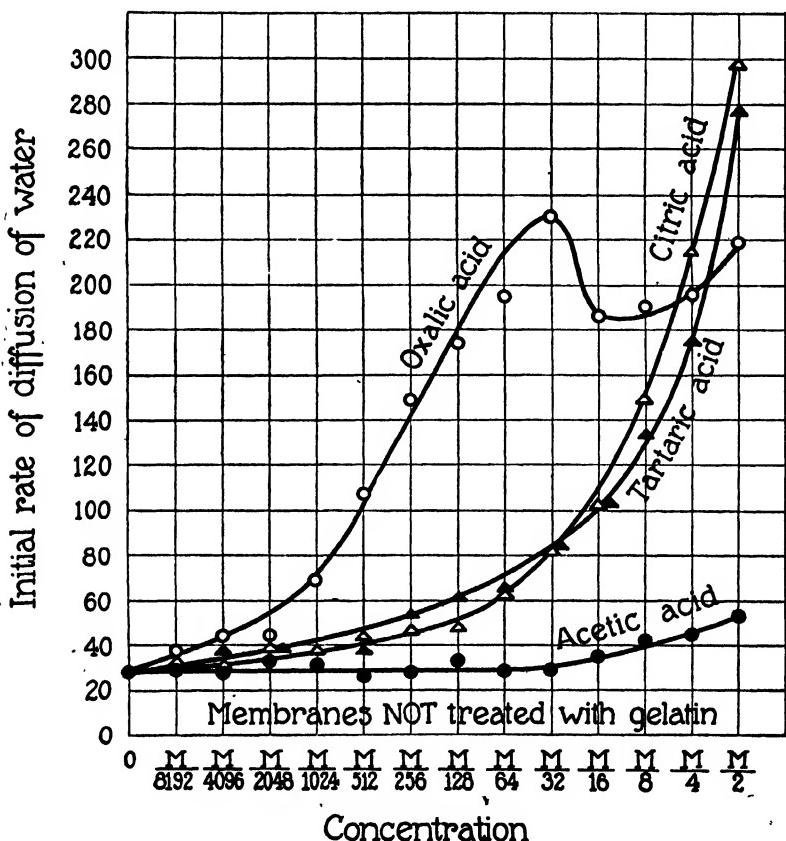


FIG. 8. Positive osmosis when solutions of the same acids as in Fig. 7 are separated from water by membranes *not* treated with gelatin.

of water are attracted more powerfully by  $\text{Na}_2\text{SO}_4$ , less powerfully by  $\text{NaCl}$ , and still less powerfully by  $\text{CaCl}_2$ . Hence when we make  $m/128$  solutions of these three salts acid through the addition of  $\text{HNO}_3$ , and if it is true that water diffuses through the membranes not treated with gelatin in the form of positively charged particles in the presence of strong acid, it should diffuse more rapidly into  $\text{Na}_2\text{SO}_4$ , less rapidly into  $\text{NaCl}$ , and least rapidly into  $\text{CaCl}_2$ .  $m/128$  solutions of these three salts were brought to a pH of 2.9 through the addition of  $\text{HNO}_3$ , and the experiment turned out as the theory demands. When the same experiment was made with collodion membranes treated with gelatin, the order of attraction of the three salts of pH 2.9 for water was reversed, water diffusing very rapidly into the acid solution of  $m/128 \text{ CaCl}_2$ , less rapidly into the acid solution of  $m/128 \text{ NaCl}$ , and still less rapidly into the acid solution of  $m/128 \text{ Na}_2\text{SO}_4$ , since in this case the particles of water diffusing through the membrane were negatively charged. When the solutions of the three salts were neutral or alkaline, no difference in the osmotic behavior of the two types of membranes was noticed.

### III.

In a previous paper<sup>1</sup> the writer had suggested that it was necessary to discriminate between the influence of cations upon the sign of electrification of water and upon the rate with which electrified water diffuses into the solution. This suggestion receives support from this paper, and it is impossible to understand the phenomenon of negative osmosis without this discrimination. Thus it is obvious that both H ions as well as trivalent cations cause the particles of water which normally have a positive charge, to assume a negative charge and it is not yet possible to state whether the H ions or the trivalent cations are more efficient in this direction. It follows, however, from the observations published in this paper that, as far as the rate of diffusion of the charged particles of water into the solution is concerned, the influence of the trivalent cations is much more powerful than that of the hydrogen ions. We express this influence in terms of electrostatic attraction and repulsion between the ions and the charged particles of water, not in order to offer thereby an explanation of this influence but merely to simplify the

presentation of the facts. With this reservation our experiments suggest that the apparent electrostatic effect of the hydrogen ions upon the motion of the electrified particles of water is small if compared with the effect of the trivalent cations; in fact it seems smaller even than that of other monovalent cations.

When acid is put into collodion bags *not* treated with gelatin, we observe a rapid diffusion of water into the acid solution as if the attraction of the positively charged water by the anion of the acid was very strong while its repulsion by the H ion was weak. When we put acids into collodion bags treated with gelatin we observe negative osmosis; *i.e.*, slight attraction of the negatively charged particles of water by the positive hydrogen ion, and a powerful repulsion of the water by the anion of the acid. In all these cases, the apparent electrostatic effect of the hydrogen ion upon the charged particles of water seems smaller than the electrostatic effect of any other ion. When we compare the electrostatic effect of trivalent cations on the rate of diffusion of water, we notice that it is much more powerful than that of the hydrogen ion. When water is negatively charged, it is attracted very powerfully by  $\text{Al}_2\text{Cl}_6$  as well as by  $\text{Al}_2(\text{SO}_4)_3$ , though naturally more by the former than by the latter. While in the case of acids the  $\text{SO}_4$  ion has a more powerful influence than the H ion, the electrostatic effect of a trivalent cation cannot be overcome by the effect of a bivalent anion. It can only be equalled by the influence of a trivalent anion; *e.g.*, the citrate. This comparatively weak electrostatic effect of the H ion is responsible for the phenomenon of negative osmosis observed in the case of acids. When we use membranes in which the electrified water is negatively charged in the presence of acid the attractive effect of the H ion is so small that the repulsive effect of the anion prevails in the critical range of concentrations, where the drop in the curve occurs. As a consequence no water can diffuse into the solution and the volume of the latter will diminish on account of the diffusion of solute into the pure solvent. Since we notice also negative osmosis in the case of  $\text{Ba}(\text{OH})_2$  and  $\text{Ca}(\text{OH})_2$ , we conclude that the electrostatic effect of the OH ion on the movable stratum of the double layer is also small.

## IV.

Gelatin is not the only substance which causes a reversal of the sign of the electrification of the particles of water diffusing through the collodion membrane in the presence of comparatively low concentrations of acid or of salts with trivalent or tetravalent cations. The same effect can be produced when the collodion membrane is treated with 1 per cent solutions of casein, egg albumin, blood albumin, and edestin; while treatment of the collodion membrane with 1 per cent solutions of Fairchild's peptone, of peptone (prepared by Dr. Northrop) from egg albumin digested with pepsin and neutralized, of alanine, of "soluble starch," of "potato starch," and of 0.50 per cent agar-agar did not cause the reversal. The proteins which modify the behavior of the membrane cannot diffuse through the latter but the writer is not yet ready to state that this is the decisive factor. It is of interest that a treatment of the collodion membrane with a 0.002 per cent solution of gelatin makes the membrane already noticeably amphoteric though to a considerably smaller degree than a treatment with a 1 per cent solution of gelatin.

## SUMMARY.

1. It is shown that collodion membranes which have received one treatment with a 1 per cent gelatin solution show for a long time (if not permanently) afterwards a different osmotic behavior from collodion membranes not treated with gelatin. This difference shows itself only towards solutions of those electrolytes which have a tendency to induce a negative electrification of the water particles diffusing through the membrane, namely solutions of acids, acid salts, and of salts with trivalent and tetravalent cations; while the osmotic behavior of the two types of membranes towards solutions of salts and alkalies, which induce a positive electrification of the water particles diffusing through the membrane, is the same.

2. When we separate solutions of salts with trivalent cation, e.g.  $\text{LaCl}_3$  or  $\text{AlCl}_3$ , from pure water by a collodion membrane treated with gelatin, water diffuses rapidly into the solution; while no water diffuses into the solution when the collodion membrane has received no gelatin treatment.

3. When we separate solutions of acid from pure water by a membrane previously treated with gelatin; negative osmosis occurs; *i.e.*, practically no water can diffuse into the solution, while the molecules of solution and some water diffuse out. When we separate solutions of acid from pure water by collodion membranes not treated with gelatin, positive osmosis will occur; *i.e.*, water will diffuse rapidly into the solution and the more rapidly the higher the valency of the anion.

4. These differences occur only in that range of concentrations of electrolytes inside of which the forces determining the rate of diffusion of water through the membrane are predominantly electrical; *i.e.*, in concentrations from 0 to about  $M/16$ . For higher concentrations of the same electrolytes, where the forces determining the rate of diffusion are molecular, the osmotic behavior of the two types of membranes is essentially the same.

5. The differences in the osmotic behavior of the two types of membranes are not due to differences in the permeability of the membranes for solutes since it is shown that acids diffuse with the same rate through both kinds of membranes.

6. It is shown that the differences in the osmotic behavior of the two types of collodion membranes towards solutions of acids and of salts with trivalent cation are due to the fact that in the presence of these electrolytes water diffuses in the form of negatively charged particles through the membranes previously treated with gelatin, and in the form of positively charged particles through collodion membranes *not* treated with gelatin.

7. A treatment of the collodion membranes with casein, egg albumin, blood albumin, or edestin affects the behavior of the membrane towards salts with trivalent or tetravalent cations and towards acids in the same way as does a treatment with gelatin; while a treatment of the membranes with peptone prepared from egg albumin, with alanine, or with starch has no such effect.



[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, January 20, 1920, Vol. ii,  
No. 3, pp. 273-296.]

## INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES ON SOME PHYSICAL PROPERTIES OF COLLOIDS AND OF CRYSTALLOIDS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 19, 1919.)

### I. INTRODUCTION.

When we separate neutral solutions of salts with monovalent cation from pure water by a collodion membrane, water will diffuse into the solution. The writer has shown in a preceding publication<sup>1</sup> that the initial rate of diffusion will at first increase with the concentration of the electrolyte, but as soon as the concentration of the latter is about  $m/256$ , the initial rate of diffusion of water into the solution will in the case of many electrolytes diminish with a further increase in the concentration of the electrolyte until at a concentration varying between  $m/32$  and  $m/8$  (according to the nature of the electrolyte) a minimum is reached. This phenomenon is due to the influence of the ions on the electrification and rate of diffusion of water through the collodion membrane. In the presence of neutral solutions of salts with monovalent or bivalent cation, water diffuses through the collodion membrane as if its particles were positively charged and as if they were attracted by the anion and repelled by the cation of the electrolyte with a force increasing with the valency of the ion. With low concentrations of electrolytes the attractive action of the anion upon the positively charged particles of water prevails over the repulsive force of the cation, while, when the concentration exceeds a certain value, which for a number of salts is about  $m/256$ , the repelling force of the cations of the electrolyte upon the positively charged particles of the water increases more rapidly than the attractive force of the anions. This idea is supported by the fact that the addition of salts with

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20 ii, 173.

bivalent cation causes a more rapid drop than the addition of a salt with monovalent cation. These phenomena bear so striking a resemblance to the action of the concentration of electrolytes upon the osmotic pressure, the swelling, and other properties of colloids that a discussion of the similarity may seem of interest.

It was found by Pauli<sup>2</sup> that the addition of a little acid to blood albumin which had been dialyzed for weeks (and which was therefore approximately isoelectric) caused an increase in the viscosity of the protein, which at first was the greater the more acid was added. Very soon, however, a point was reached where the addition of more acid caused again a diminution in the viscosity. The same phenomenon occurs when acid is added to isoelectric gelatin. The addition of a slight amount of acid causes an increase in the osmotic pressure until finally a point is reached where the further addition causes a diminution (Fig. 1). The increase in the osmotic pressure of isoelectric gelatin when a slight quantity of HCl is added, is due to the formation of gelatin chloride, but the depressing effect of the addition of an excess of acid is not so easy to explain. According to Pauli we should ascribe it to the diminution of the degree of electrolytic dissociation of protein chloride due to the increase in the concentration of the common anion Cl. The writer's measurements of conductivity do not support this idea.<sup>3</sup> Another suggestion made by colloid chemists is that the addition of more acid causes an aggregate formation of the gelatin particles and therefore a diminution of osmotic pressure. This suggestion rests only on the phenomenon which it is supposed to explain, but it may, nevertheless, be correct. If so, it remains to be explained why an increase in the concentration of electrolytes causes a formation of aggregates.

In a preceding publication<sup>4</sup> the writer has shown that a 1 per cent solution of gelatin-acid salt, e.g. gelatin chloride or gelatin citrate, etc., has its maximal osmotic pressure when the pH is about 3.4 or 3.3. When to gelatin chloride of this pH acid or neutral salt is added, the osmotic pressure (as well as the swelling, viscosity, etc.) falls and the more so the more acid or salt has been added. When we add

<sup>2</sup> Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

<sup>3</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 483, 559.

<sup>4</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 559.

alkali, the osmotic pressure falls also, but in this case a complication arises, since the addition of NaOH to a gelatin-acid salt causes a neutralization of the acid and the gradual transformation of the gelatin-acid salt into isoelectric gelatin which has a minimal osmotic pressure.

A 1 per cent solution of metal gelatinate; *e.g.* Na gelatinate, has its maximal osmotic pressure at a pH of about 8.4. When to a 1 per cent solution of metal gelatinate of this pH alkali or neutral salt is added, the osmotic pressure (as well as the swelling and the viscosity) is diminished. When acid is added the same phenomenon occurs but for another reason since the addition of acid lowers the pH and transforms gelatin salt into isoelectric gelatin. We will first discuss the action of electrolytes on the osmotic pressure of metal gelatinate.

## *II. Water Charged Positively.*

Doses of 1 gm. each of commercial, finely powdered gelatin are rendered isoelectric, melted, and made up into 1 per cent solutions of gelatin by adding enough NaOH and water to make the volume 100 cc. The amount of NaOH contained in 100 cc. of each solution varied and it was for different solutions 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of  $\text{M}/4$  NaOH. Part of this NaOH combined with the gelatin, forming Na gelatinate; the rest remained free. As shown in a preceding paper, there exists a definite chemical equilibrium between the Na gelatinate formed, the isoelectric gelatin, and the hydrogen ion concentration. If the alkali of such a solution is neutralized by an acid (*e.g.*  $\text{CO}_2$ ) or if it is allowed to diffuse out from the solution, the equilibrium is disturbed and some of the metal gelatinate will be transformed into non-ionogenic (isoelectric) gelatin.

These gelatin solutions containing different amounts of NaOH were put into collodion bags. The latter were closed with a rubber stopper which was perforated by a glass tube with a bore of 2 mm. in diameter which served as a manometer to measure the osmotic pressure of the solutions. These bags were put into beakers containing 350 cc. of a solution of NaOH which in each case had the same amount of NaOH in 100 cc. solution as was originally added to the gelatin solution which it surrounded. Thus the 1 per cent solution of Na

gelatinate which had 6.4 cc. of m/4 NaOH in 100 cc. was dipped into a beaker with water which had 6.4 cc. of m/4 NaOH per 100 cc. of H<sub>2</sub>O. Since a small part of the NaOH inside the collodion bag had combined with the gelatin, the concentration of NaOH in the outside solution was at first slightly greater than that inside, and as a consequence some NaOH diffused from the outside into the bags. Part of the NaOH in the outside solution was gradually neutralized by

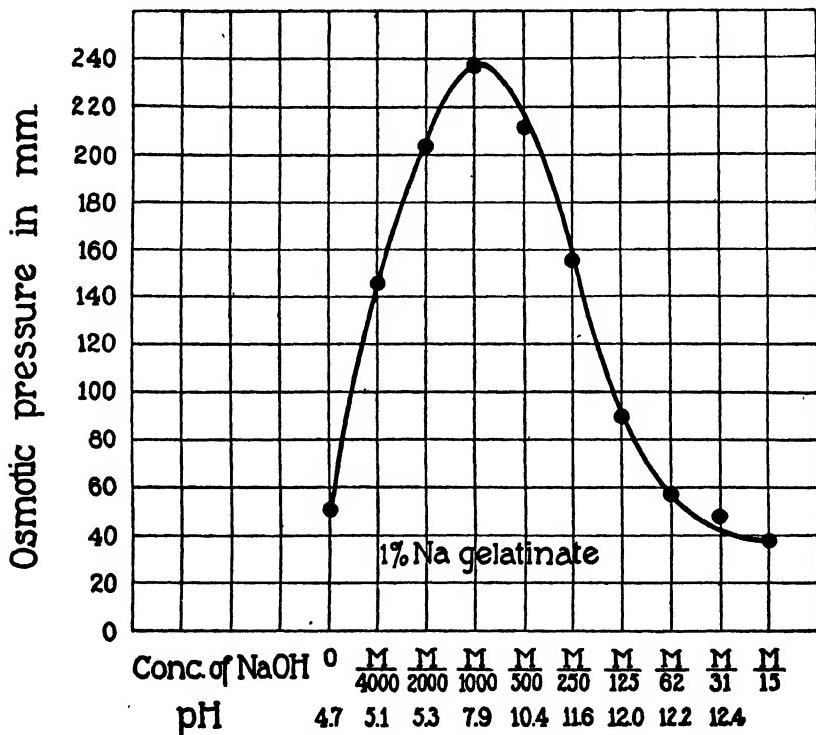


FIG. 1. Curve of osmotic pressure of 1 per cent Na gelatinate solutions contained in collodion bags and surrounded by solutions of NaOH of the same concentration as that in which the isoelectric gelatin was dissolved. Abscissæ are the logarithms of concentration of NaOH in which the isoelectric gelatin was dissolved; below is given the pH of the gelatin solution at the conclusion of the experiment (4½ hours after beginning). The osmotic pressure rises steeply at first until the pH is about 8.4, and then when more NaOH is contained in solution the osmotic pressure falls again equally steeply with a further increase in concentration of NaOH added.

the absorption of  $\text{CO}_2$  from the air and this diminished the concentration of  $\text{NaOH}$  in the outside and, as a consequence, also in the inside solution.

Fig. 1 represents the osmotic pressure reached after about 5 hours in the various solutions. (At this time the permanent osmotic pressure is generally attained when the solution undergoes no further chemical changes.) The abscissæ of Fig. 1 are the logarithms of the concentration of the  $\text{NaOH}$ . The row of figures below the figures for the concentration of  $\text{NaOH}$  is the pH as found in each gelatin solution at the end of the experiment. The ordinates are the osmotic

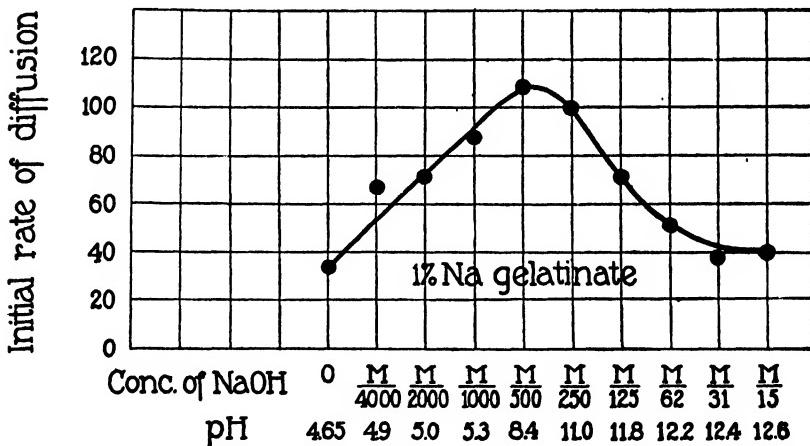


FIG. 2. Curve of initial rate of diffusion of water into 1 per cent gelatin dissolved in the same concentrations of  $\text{NaOH}$  as those used in Fig. 1, measured by rise of level of liquid in manometer 30 minutes after beginning of experiment. Abscissæ are the concentrations of  $\text{NaOH}$ , ordinates are the rise of level of liquid in manometer after 30 minutes. The curve is similar to the curve of osmotic pressure. Maximum at pH = 8.4.

pressures expressed in mm. of a column of 1 per cent gelatin solution. The maximum should have been at a pH of about 8.4 (between  $\text{M}/1,000$  and  $\text{M}/500$ ). The figure shows that the osmotic pressure rises first rapidly with an increase in the pH and falls equally rapidly beyond a pH which (from other experiments) we judge to be about 8.4. At a pH of 12.2 the curve is almost as low as it is for isoelectric gelatin.

Fig. 2 shows the influence of the concentration of NaOH upon the initial rate of diffusion of water into the solutions of Na gelatinate. The ordinates represent the height to which the liquid had risen in the manometer 30 minutes after commencement of the experiment. The abscissæ are again the logarithms of the concentration of the NaOH and the pH is that found after 30 minutes. The reader will notice that the critical points in this curve coincide with the critical points in Fig. 1. The curve rises steeply until pH = 8.4 when the maximum is reached, and falls equally steeply until a pH = 12.2 is reached. We have given the reason for this coincidence of the critical points in the curves of Figs. 1 and 2 in a preceding paper.<sup>5</sup>

The question arises, what causes the drop of the two curves when the pH exceeds 8.4? This drop seems to be the same as the drop in the initial rate of diffusion of water into a solution of  $\text{Na}_2\text{SO}_4$  which occurs when the concentration exceeds  $\text{M}/256$ . When we separate metal gelatinates from distilled water by a collodion membrane, water diffuses into the gelatin solution as if its particles were positively charged; being attracted by the gelatin anion and repelled by the metal ion. Metal gelatinates behave therefore towards the electrification and rate of diffusion of water through collodion membranes like neutral or alkaline solutions of  $\text{Na}_2\text{SO}_4$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$ . The analogy can be carried further, since the addition of little salt depresses both the rate at which water will diffuse into the gelatin solution through the membrane as well as the permanent osmotic pressure of the solution.

Fig. 3 shows the rapid fall in the permanent osmotic pressure of a 1 per cent solution of Na gelatinate with a pH of about 8.4 to which various concentrations of a salt, KCl,  $\text{K}_2\text{SO}_4$ ,  $\text{K}_3$  citrate, and  $\text{CaCl}_2$ , are added. The amounts of salts contained in 100 cc. of gelatin solution were 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of  $\text{M}/4$ . These solutions were put into the collodion bags described, and the collodion bags were put into beakers containing the same salt in the same concentration which had been added to the gelatin solution. Fig. 3 gives the rise in the manometer after 30 minutes and Fig. 4 the osmotic pressure after 6 hours. The curves show the rapid drop

<sup>5</sup> Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 87.

of the initial rate of diffusion, as well as of the permanent osmotic pressure with the increase in the concentration of the salt added. The drop is more rapid when Ca is added than when K is added, as it should be on account of the fact that the particles of water diffusing through the membrane are positively charged.

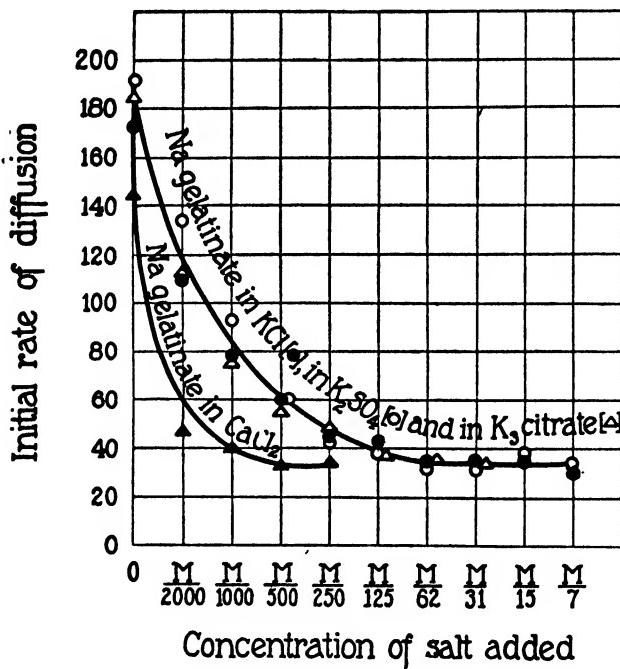


FIG. 3. Depressing effect of the addition of neutral salt to a 1 per cent solution of Na gelatinate of pH about 8.4 upon the initial rate of diffusion of water into the solution during 30 minutes. Abscissæ are the concentration of salt added, ordinates the height of level of liquid in manometer after 30 minutes. The depressing effect of Ca is greater than that of K.

Figs. 5 and 6 show that the depressing effect of the addition of alkali is the same as that of the addition of neutral salt with the same cation,  $\text{Ca}(\text{OH})_2$ , causing a greater depression than KOH. This contradicts the statement current in the literature of colloid chemistry that salts lower and that alkalies raise the osmotic pressure of gelatin solutions.

The influence of the concentration of electrolytes upon the electrification and rate of diffusion of water into gelatin solutions is similar

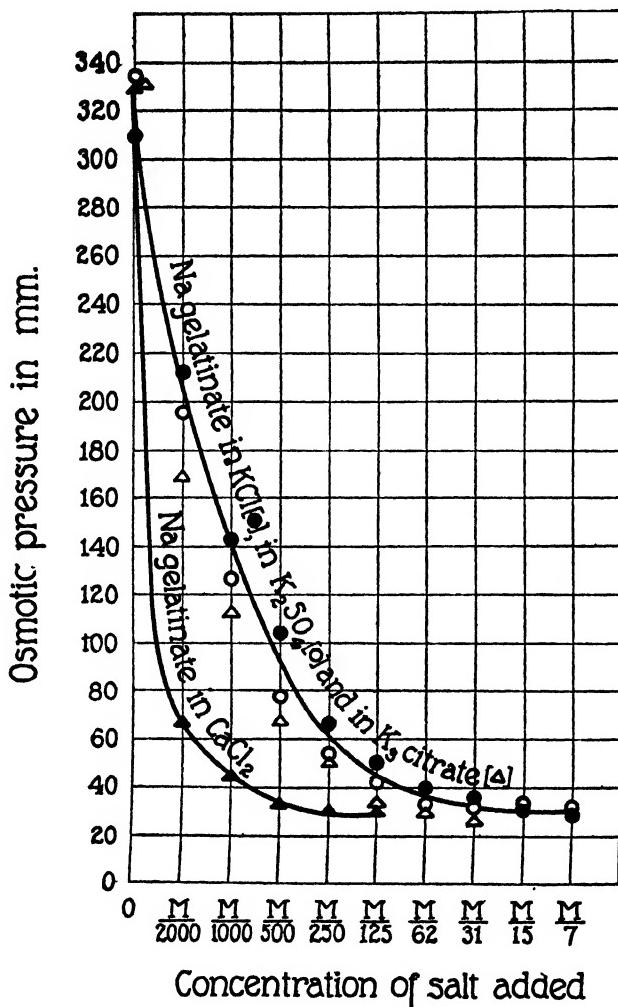


FIG. 4. Depressing effect of the addition of salts to 1 per cent Na gelatinate of pH = 8.4 upon osmotic pressure of solution. Ca depresses more than K.

to the influence of the concentration of electrolytes when we substitute a neutral salt like  $\text{Na}_2\text{SO}_4$  or  $\text{Na}_4\text{Fe}(\text{CN})_6$  for the solution of metal

gelatinate. It may be well to point out this analogy by making the experimental methods in both cases as much alike as possible.

To solutions of  $M/256 Na_2SO_4$  were added the same concentrations of salt as in the gelatin experiments; namely, 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of  $M/4$  salt in 100 cc. of solution. These solutions were put into collodion bags and the latter were put into the solutions of the same salts as those in which the  $Na_2SO_4$  was made

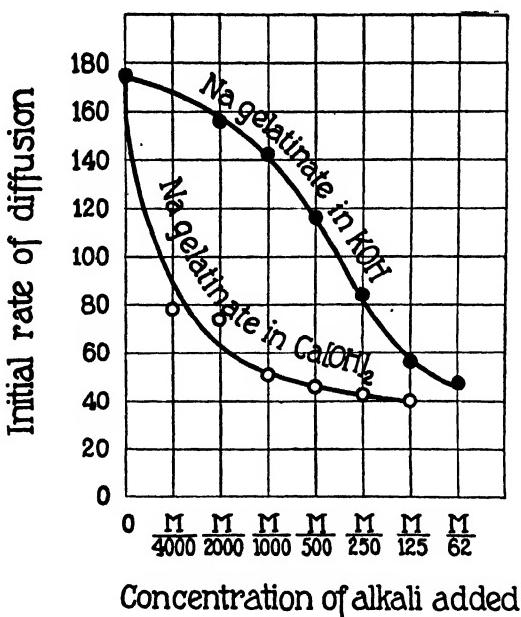


FIG. 5. Depressing effect of the addition of alkali to 1 per cent  $Na$  gelatinate of  $pH = 8.4$  upon the initial rate of diffusion of water into the gelatin solution.  $Ca(OH)_2$  depresses more than  $KOH$ .

up. This means, that when 100 cc. of the solution in the collodion bag were a mixture of  $M/256 Na_2SO_4$  in  $M/2,000 KCl$ , the outside solution was  $M/2,000 KCl$  (without  $Na_2SO_4$ ), and so on. This was done to make the experiments in every point like the gelatin experiments, in which the outside solution also contained the same concentration of salt which was added to the gelatin solution. It was found that the addition of salts and of alkali depresses the initial rate of

diffusion of water into a M/256 solution of  $\text{Na}_2\text{SO}_4$  in the same way as it depresses the initial rate of diffusion of water into a solution of Na gelatinate.

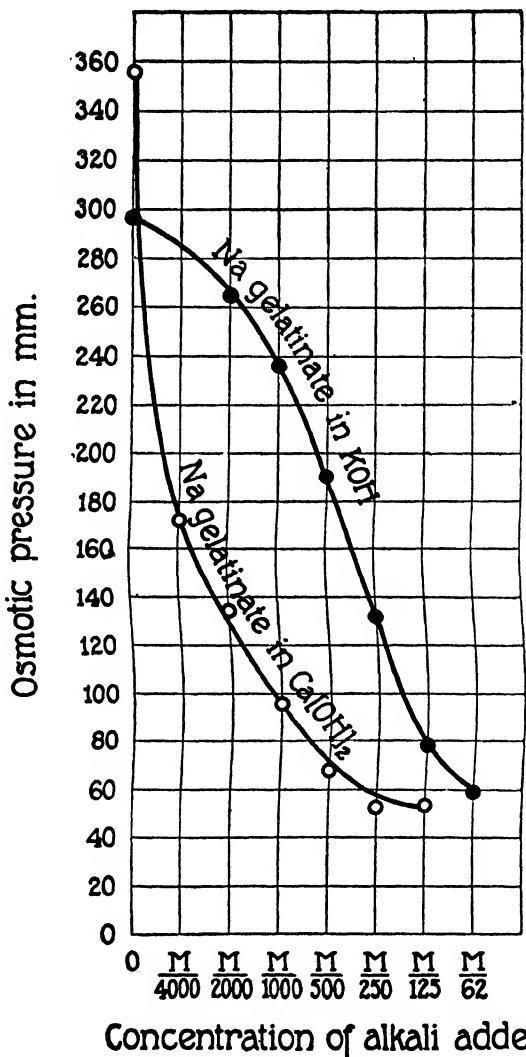


FIG. 6. Depressing effect of the addition of alkali to 1 per cent Na gelatinate of pH = 8.4 upon osmotic pressure of gelatin solution.  $\text{Ca}(\text{OH})_2$  depresses more than KOH. The effect is similar to that of addition of neutral salt.

This is illustrated by the experiments represented in Fig. 7. The abscissæ are the concentration of the salt added to the M/256  $\text{Na}_2\text{SO}_4$  solutions. The electrolytes added were KOH, KCl, and  $\text{MgCl}_2$ .

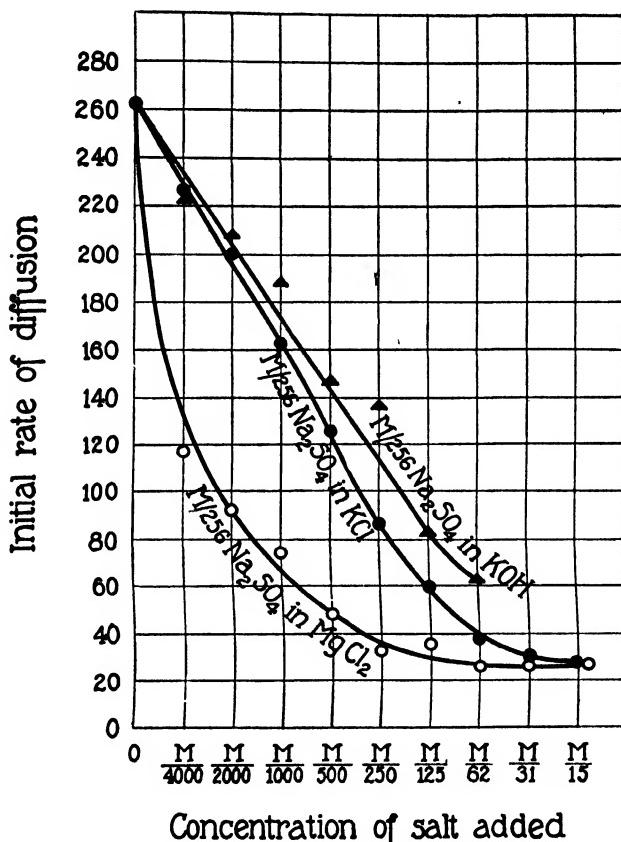


FIG. 7. M/256  $\text{Na}_2\text{SO}_4$  solutions separated from water by a collodion membrane, showing that the addition of salt or alkali to M/256  $\text{Na}_2\text{SO}_4$  causes a similar depression upon the initial rate of diffusion of water into the solution of M/256  $\text{Na}_2\text{SO}_4$  as the addition of salt to a solution of Na gelatinate. Mg depresses more than K.

There was little difference in the effect of KOH and of KCl, while the depressing effect of  $\text{MgCl}_2$  was considerably greater. It should be stated that the collodion membranes used in these experiments had not been treated with gelatin.

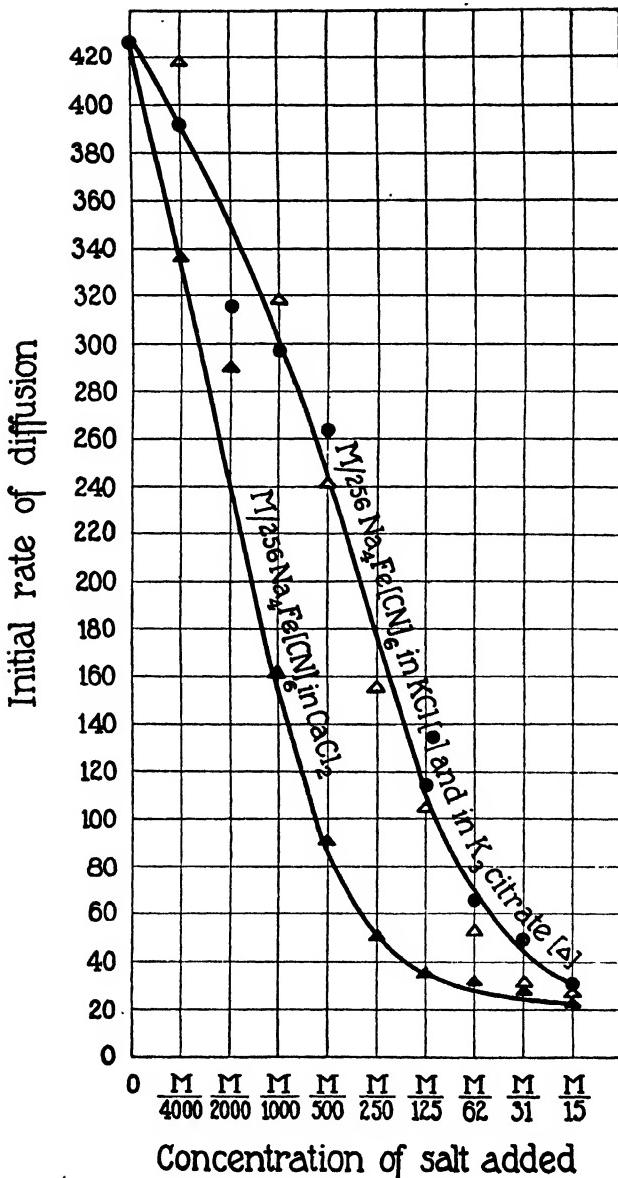


FIG. 8.  $M/256\text{Na}_4\text{Fe}(\text{CN})_6$  separated from water by a collodion membrane, showing the depressing effect of the addition of salt to the solution upon the initial rate of diffusion of water into the solution.

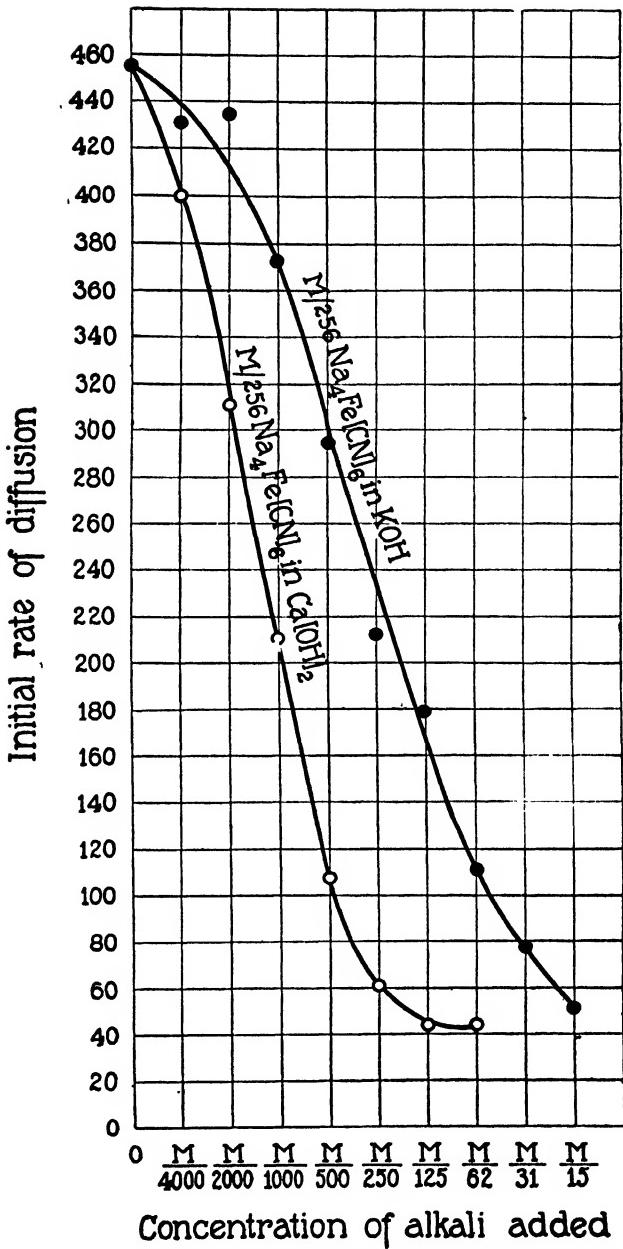


FIG. 9. Showing the same depressing effect of the addition of alkali to m/256  $\text{Na}_4\text{Fe}(\text{CN})_6$ .

These experiments can be repeated with any other neutral or alkaline salt with univalent cation. Figs. 8 and 9 show the depressing effect of the addition of neutral salts (Fig. 8) or of alkalies (Fig. 9) to a solution of M/256  $\text{Na}_4\text{Fe}(\text{CN})_6$ . Again the influence of the valency of the cation and the identity of the effects of alkalies and neutral salts with the same cation are manifest.

In all these experiments in which the particles of water were positively charged the depressing effect of the addition of salt was greater when the cation of the salt was bivalent than when it was monovalent.

Such experiments have been made with a large number of salts, all yielding the same result; namely, that the depressing effect of the addition of increasing concentrations of alkalies and neutral salts upon the attraction of water by gelatin solutions is paralleled by the influence of the addition of increasing concentrations of salts upon the attraction of water by solutions of salts, in the presence of which water is positively charged. This raises the question whether or not the depressing influence of high concentrations of electrolytes upon the osmotic pressure of gelatin is necessarily connected with the colloidal character of gelatin or whether it is based upon a much more general property of matter; namely, the influence of electrolytes upon the electrification and the rate of diffusion of water through membranes. We only wish to point out this possibility without deciding definitely.

It may suffice to point out that the influence of the concentration and the valency of electrolytes on the osmotic pressure of gelatin solutions can be demonstrated equally well for the phenomenon of swelling and the curves representing this influence are similar to those given in this paper for the influence on the osmotic pressure.

### *III. Negatively Charged Particles of Water.*

When we separate solutions of gelatin-acid salts from pure water by a collodion membrane, the particles of water diffusing through the membrane act as if they were negatively charged. That this is so can be demonstrated by experiments on electrical endosmose.

When acid, *e.g.* HCl, is added to isoelectric gelatin, part of the acid combines with gelatin to form gelatin chloride and part of the acid remains free. There is a chemical equilibrium between isoelectric gelatin, gelatin chloride, and free hydrochloric acid. As long as the pH of the gelatin chloride solution does not exceed 3.3 (*i.e.* as long as the pH varies between 4.7 and 3.3) the osmotic pressure of gelatin chloride increases with an increase in the concentration of the acid. As soon, however, as the pH reaches 3.3, the osmotic pressure of gelatin chloride diminishes again with increasing concentration of acid.

Fig. 10 may serve as an illustration. To each of a series of doses of 1 gm. of isoelectric gelatin were added 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of  $m/4$  HCl, water was added, the gelatin melted by heating to about 40°C., and enough water was added to make the volume of the solution 100 cc., so that each solution contained 1 per cent gelatin. This solution was put into collodion bags closed by a rubber stopper perforated with a glass tube serving as a manometer. The collodion bag was put into a beaker containing 350 cc. of the same concentration of acid in water as that originally added to the gelatin. Thus the gelatin solution to which 0.2 cc. of  $m/4$  HCl was added per 100 cc. of solution was immersed in a HCl solution containing 0.2 cc. of  $m/4$  HCl in 100 cc. of water. This outside solution was a little more concentrated than the concentration of the free acid inside, since part of the acid added to the gelatin entered into combination with the latter. This difference was equalized by the diffusion of some of the outside acid into the gelatin solution, thus slightly lowering the original pH.

The upper curve (Fig. 10) shows that the osmotic pressure of the solution (measured after 20 hours) rises with an increase of the hydrogen ion concentration until it reaches a maximum at pH about 3.4 or 3.3, and that with a further rise in the amount of free acid the osmotic pressure of the solution falls until at pH 1.75 the osmotic pressure of the gelatin solution is almost as low as it is near the isoelectric point.

The lower curve in Fig. 10 represents the velocity of diffusion of water into the gelatin solution measured by the height of the column of liquid in the manometer after 30 minutes. The maximum and the two minima of the curve coincide with those of the upper curve

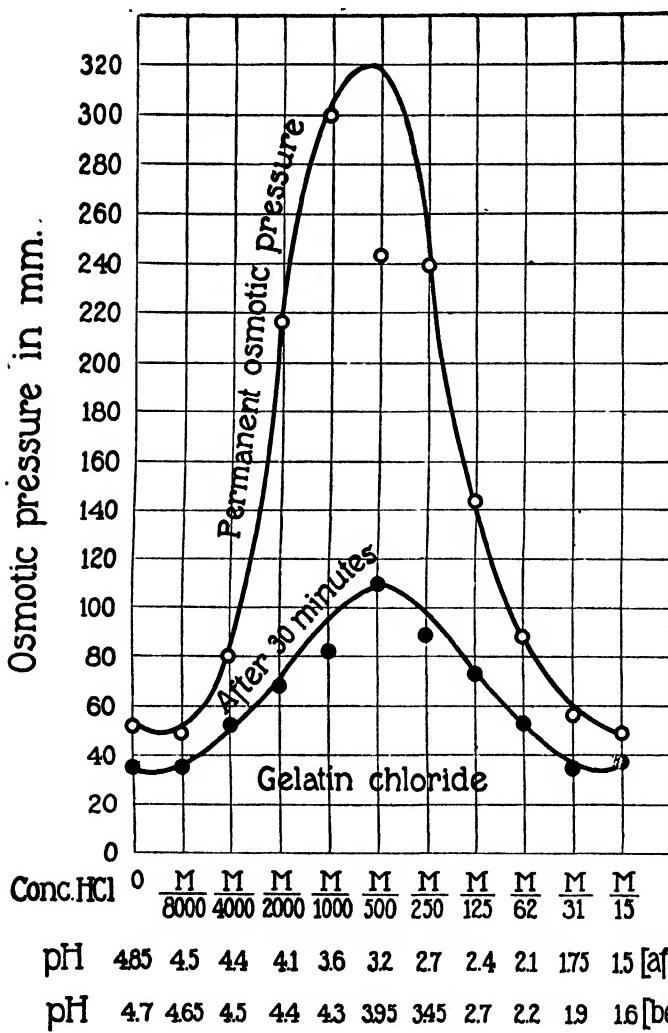


FIG. 10. 1 per cent isoelectric gelatin solutions dissolved in increasing concentrations of HCl. Abscissæ are the concentrations of HCl in which the gelatin is dissolved, with the pH at the end and at the beginning of experiment. Ordinates of upper curve represent the final osmotic pressure (after 20 hours); ordinates of lower curve represent the initial rate of diffusion of water into solution. Maximum in both cases at pH 3.3. Showing the depressing effect of the further addition of HCl when pH 3.3 is reached.

representing the permanent osmotic pressures of the various gelatin solutions.

When we select gelatin chloride with about the maximal osmotic pressure, *i.e.* gelatin chloride with a pH of 3.5, and add various

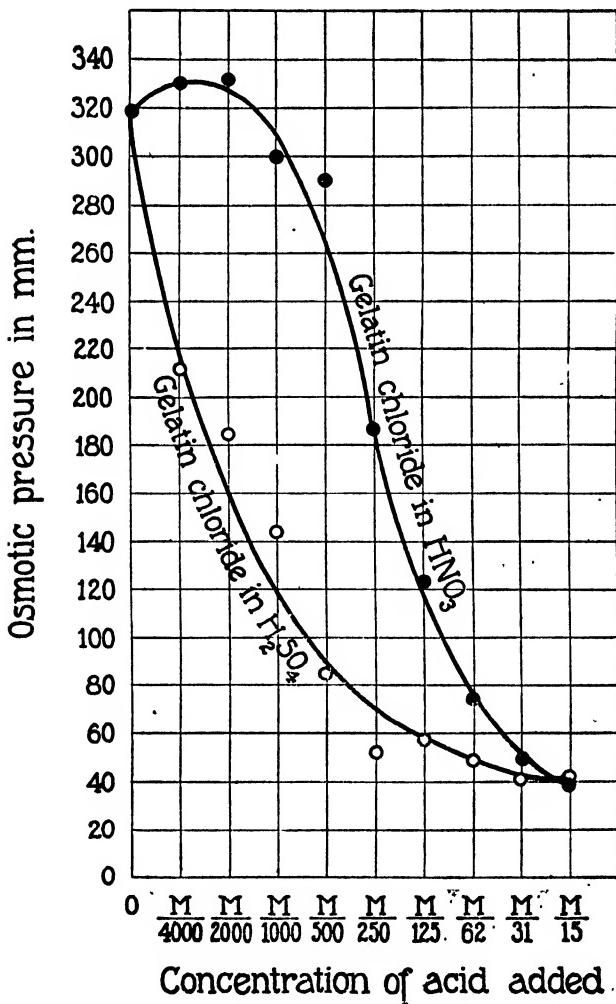


FIG. 11. Showing depressing effect of addition of  $\text{HNO}_3$  and  $\text{H}_2\text{SO}_4$  to 1 per cent gelatin chloride upon osmotic pressure. Depressing effect of  $\text{SO}_4$  greater than that of  $\text{HNO}_3$ .

concentrations of another acid to it, we notice a similar depression. Fig. 11 gives the effect of the addition of different quantities of  $\text{HNO}_3$  and of  $\text{H}_2\text{SO}_4$  to 1 gm. of gelatin of pH 3.3; 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of  $\text{M}/4$  acid were contained in 100 cc. of gelatin solution of pH 3.5. The outside solution contained the same concentration of acid. The depressing effect of  $\text{HNO}_3$  was like that of  $\text{HCl}$ , and the depressing effect of  $\text{H}_2\text{SO}_4$  was greater. Fig. 12 shows that the depressing influence of the addition of acid is manifested in a similar way in the influence of the concentration of acid upon the initial diffusion of water into the gelatin solution.

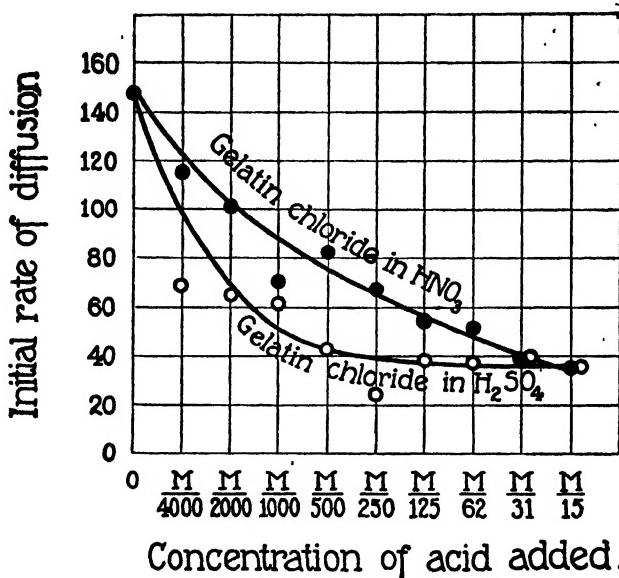
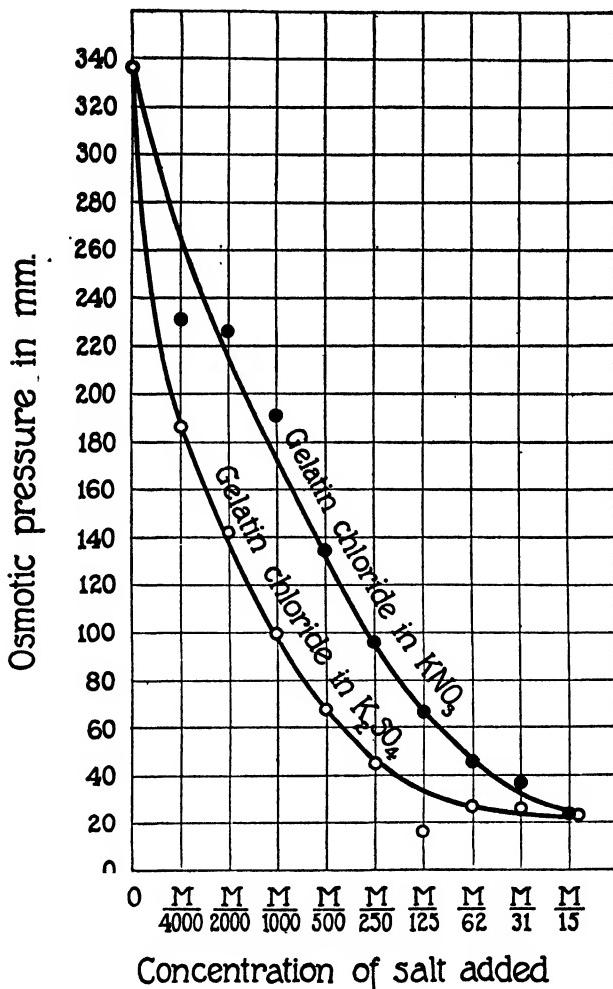


FIG. 12. The same effect of the addition of acid upon the initial rate of diffusion of water into the gelatin chloride solution.

If we add neutral salt, e.g.  $\text{KNO}_3$  or  $\text{K}_2\text{SO}_4$ , to a 1 per cent gelatin solution with a pH = 3.5, taking care that the pH of the solution is not altered by the addition of salt, the depressing effect is about the same as when we add acid. To doses of 1 gm. isoelectric gelatin of pH 3.5 were added 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of  $\text{M}/4$   $\text{KNO}_3$  or  $\text{K}_2\text{SO}_4$ , and enough water was added to make the volume 100 cc. The pH remained the same in all solutions.

The outside solutions had the same pH as the solution in the colloidion bag and contained also the same concentration of potassium

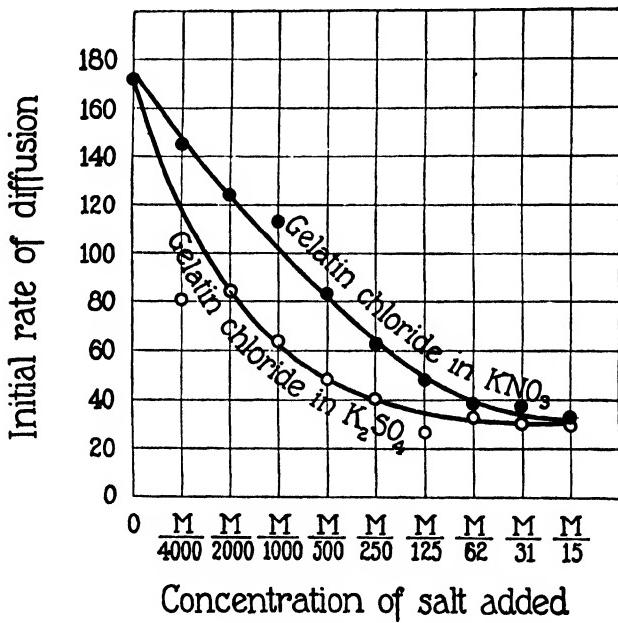


FIGS. 13 and 14. Depressing effect of addition of salt to 1 per cent gelatin chloride pH = 3.3.  $\text{SO}_4$  depresses more than  $\text{NO}_3$ .

salt as was added to the gelatin. The curves in Figs. 13 and 14 show that the depressing effect of an addition of  $\text{KNO}_3$  is about the same as the addition of an equal amount of  $\text{HNO}_3$ , and that the addition

of  $K_2SO_4$  has about the same depressing effect as the addition of  $H_2SO_4$ .

When gelatin-acid salts, *e.g.* gelatin chloride, are separated from distilled water or a salt solution of the same pH by a collodion bag, water diffuses through the membrane in the form of negatively charged particles which are attracted by the gelatin cation and repelled by the anion. When we wish to replace the gelatin solution by a crystalloidal electrolyte in the presence of which water diffuses



of the collodion membrane with a protein is necessary since otherwise water will diffuse through the collodion membrane in the form of positively charged particles and then the case is no longer comparable with that of gelatin-acid salts. This gelatin treatment of the collodion membrane is not required when we wish to experiment with positively charged particles of water, since water assumes practically always a positive charge when in contact with a collodion membrane free from gelatin, even in comparatively high concentrations of acid.

If we use membranes which had contained a 1 per cent gelatin solution over night but were then freed from the gelatin by a number of washings with warm water, and if we substitute for the gelatin-acid salt a crystalloid salt which causes water to be charged negatively in such gelatin-treated membranes, *e.g.*  $M/512 Al_2Cl_6$ , we notice the same depressing effect of an increase of the concentration of electrolytes on the initial rate of diffusion of water into the solution as was described in the case of the solutions of gelatin-acid salts. When we separate a collodion bag filled with  $M/512 Al_2Cl_6$  (which causes water to be charged negatively) and put it into  $H_2O$ , the level of liquid in the manometer will rise to a height of about 300 mm. in 20 minutes. When we add varying quantities of a neutral salt to the  $M/512$  solution of  $Al_2Cl_6$ , and put the same concentration of salt into the outside beaker containing the water—in order to eliminate the direct effect of the salt added upon the rate of diffusion—we notice that the rate of diffusion of water will diminish the more rapidly the more salt we add. This is illustrated in Fig. 15.  $NaNO_3$  and  $CaCl_2$  have about the same depressing effect, showing that the depression is not due to the influence of the cation; while  $SO_4$  and still more citrate depress much more powerfully than the chlorides, showing that the depression is due to the influence of the increasing concentration of the anion upon the negatively charged particles of water.

Similar experiments were made with solutions of  $CaCl_2$  which were rendered sufficiently acid in order to cause the water to be charged negatively. If we select  $M/128$  solutions of such acidulated  $CaCl_2$  as a substitute for gelatin-acid solutions, we find that the addition of acid as well as of neutral salt causes a depression of the influence of

the acidulated  $\text{CaCl}_2$  solution upon the initial rate of diffusion of water into the solution.

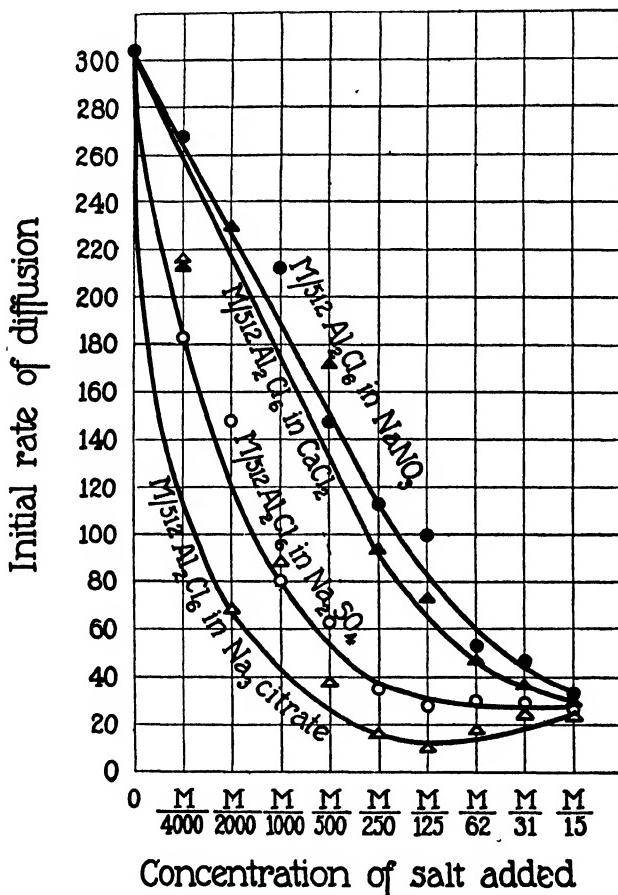


FIG. 15.  $\text{m}/512 \text{ Al}_2\text{Cl}_6$  separated from water by collodion bag. Depressing effect of addition of salt to  $\text{m}/512 \text{ Al}_2\text{Cl}_6$  upon the initial rate of diffusion of water into the bag.  $\text{SO}_4$  depresses more than  $\text{NO}_3$  or  $\text{Cl}$ .

In all the experiments mentioned the electrolyte was added not only to the inside but also to the outside solution. It is hardly necessary to state that the depressing effect is also well marked if the electrolyte is added only to the solution inside the collodion bag, while on the outside is distilled water.

These facts, then, show that the depressing effect of the addition of electrolyte upon the osmotic pressure of gelatin solutions (or colloidal solutions in general) is paralleled by the effect the addition of electrolyte has upon the initial rate of diffusion from water into solution through a collodion membrane, when we substitute a crystalloidal electrolyte for the gelatin salt.

#### SUMMARY.

1. When a 1 per cent solution of a metal gelatinate, *e.g.* Na gelatinate, of pH = 8.4 is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain rate which can be measured by the rise of the level of the liquid in a manometer. When to such a solution alkali or neutral salt is added the initial rate with which water will diffuse into the solution is diminished and the more so the more alkali or salt is added. This depressing effect of the addition of alkali and neutral salt is greater when the cation of the electrolyte added is bivalent than when it is monovalent. This seems to indicate that the depressing effect is due to the cation of the electrolyte added.

2. When a neutral M/256 solution of a salt with monovalent cation (*e.g.* Na<sub>2</sub>SO<sub>4</sub> or K<sub>4</sub>Fe(CN)<sub>6</sub>, etc.) is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain initial rate. When to such a solution alkali or neutral salt is added, the initial rate with which water will diffuse into the solution is diminished and the more so the more alkali or salt is added. The depressing effect of the addition of alkali or neutral salt is greater when the cation of the electrolyte added is bivalent than when it is monovalent. This seems to indicate that the depressing effect is due to the cation of the electrolyte added. The membranes used in these experiments were not treated with gelatin.

3. It can be shown that water diffuses through the collodion membrane in the form of positively charged particles under the conditions mentioned in (1) and (2). In the case of diffusion of water into a neutral solution of a salt with monovalent or bivalent cation the effect of the addition of electrolyte on the rate of diffusion can be explained on the basis of the influence of the ions on the electrifica-

tion and the rate of diffusion of electrified particles of water. Since the influence of the addition of electrolyte seems to be the same in the case of solutions of metal gelatinate, the question arises whether this influence of the addition of electrolyte cannot also be explained in the same way, and, if this be true, the further question can be raised whether this depressing effect necessarily depends upon the colloidal character of the gelatin solution, or whether we are not dealing in both cases with the same property of matter; namely, the influence of ions on the electrification and rate of diffusion of water through a membrane.

4. It can be shown that the curve representing the influence of the concentration of electrolyte on the initial rate of diffusion of water from solvent into the solution through the membrane is similar to the curve representing the permanent osmotic pressure of the gelatin solution. The question which has been raised in (3) should then apply also to the influence of the concentration of ions upon the osmotic pressure and perhaps other physical properties of gelatin which depend in a similar way upon the concentration of electrolyte added; *e.g.*, swelling.

5. When a 1 per cent solution of a gelatin-acid salt, *e.g.* gelatin chloride, of pH 3.4 is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain rate. When to such a solution acid or neutral salt is added—taking care in the latter case that the pH is not altered—the initial rate with which water will diffuse into the solution is diminished and the more so the more acid or salt is added. Water diffuses into a gelatin chloride solution through a collodion membrane in the form of negatively charged particles.

6. When we replace the gelatin-acid salt by a crystalloidal salt, which causes the water to diffuse through the collodion membrane in the form of negatively charged particles, *e.g.*  $m/512 Al_2Cl_6$ , we find that the addition of acid or of neutral salt will diminish the initial rate with which water diffuses into the  $m/512$  solution of  $Al_2Cl_6$ , in a similar way as it does in the case of a solution of a gelatin-acid salt.

## QUANTITATIVE LAWS IN REGENERATION. I.

By JACQUES LCEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 22, 1919.)

Regeneration consists in the growth of resting cells or buds as a consequence of the removal of parts of the body. Two problems are, therefore, involved in this phenomenon; namely, first, the quantity of growth which is measured by the mass of the regenerated organs, and, second, the problem of inhibition, or the fact that regeneration or growth of the resting cells or buds does not take place without removal of part of the body.

### *I. The Law of Direct Proportionality between the Mass of Sister Leaves of *Bryophyllum* and the Mass of Roots and Shoots Produced by Them.*

The writer had already shown that equal masses of sister leaves of *Bryophyllum calycinum* when isolated from the rest of the plant produce in equal time equal masses of shoots under equal conditions of temperature, moisture, and illumination.<sup>1</sup> The following measurements show that the same law holds also for the production of roots. In order to be able to measure the mass of roots correctly, it was necessary to let the roots develop in water. Part of a leaf was dipped into water, and in the wetted notches shoots and roots grew out rapidly, thereby preventing the growth of roots and shoots from the non-submerged notches of the leaf. In order to get accurate measurements of root production it was necessary to determine the dry weight of the roots. In addition, the dry weight of the leaves and of the shoots was also determined. The experiments lasted usually from 4 to 5 weeks in order to get larger masses of roots and to diminish the error in determining the quantity of regeneration. The organs were dried in an oven at a temperature of between 100° and 110°C. for about 24 hours.

<sup>1</sup> Loeb, J., *Bot. Gaz.*, 1918, lxv, 150.

Five pairs of sister leaves were suspended sidewise, the lower edge dipping in water, and roots and shoots formed at the lower edges only. Table I gives the dry weights of the leaves, of the shoots, and of the roots at the end of the experiment. The figure in parentheses, behind the dry weight of the shoots, expresses the number of shoots formed. The first horizontal row gives the dry weights of these organs for one set of five leaves (Set I), the second horizontal row gives the dry weights of these organs for the five sister leaves (Set II). The third horizontal row gives the ratio of the dry weights of the two sets of organs. The masses of the two sets of sister leaves were almost alike, and the ratio of the two masses was 1.02. According to the law expressed in the writer's former publications the

TABLE I.  
*Duration of Experiment 34 Days.*

	Dry weight of		
	Leaves.	Shoots.	Roots.
	gm.	gm.	gm.
Set I.....	1.755	0.247 (14)	0.113
" II.....	1.718	0.247 (15)	0.120
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$ .....	1.02	1.00	0.94

mass of shoots and of roots produced by the two sets of leaves should also be alike (within the limits of accuracy of these experiments). The ratio of the two masses of shoots and roots should, therefore, have been approximately 1.00.

The figures of the third row show that the ratio of the dry weights of the two sets of shoots is exactly 1.00 and the ratio of the two sets of roots is 0.94. *The two sets of sister leaves having equal mass produced, during the same time and under equal conditions of temperature, moisture, and illumination, equal masses of shoots and of roots.*

In the next experiment one set of six isolated leaves (Set II) remained intact while the mass of each leaf of the second set was reduced to approximately one-half by cutting away one side of each leaf (Set I) (Fig. 1). The masses of the two sets of leaves were

therefore no longer equal but had approximately the ratio of about 1:2. It was to be expected that the dry weights of the shoots and roots produced by the two sets of leaves should also be in the ratio of about 1:2, and this was the case (within the limits of the possible accuracy of such experiments) (Table II). The ratio of the dry weights of the two sets of leaves was 0.54, the ratio of the weights

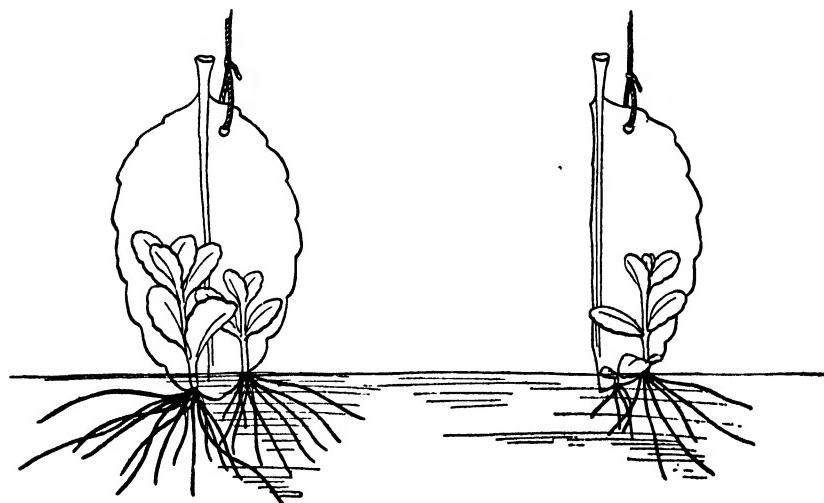


FIG. 1. Root and shoot formation in whole leaf and in sister leaf (from which the left half is cut off) dipping with their apices in water. Root and shoot formation only in the wetted parts of leaf. Diagrammatic.

TABLE II.  
*Duration of Experiment 30 Days.*

	Dry weight of		
	Leaves. gm.	Shoots. gm.	Roots. gm.
Set I (six half leaves).....	1.245	0.174 (10)	0.054
" II (" whole " ).....	2.300	0.283 (16)	0.092
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$ .....	0.54	0.61	0.59

of shoots produced by them was 0.61, and the ratio of roots produced was 0.59. The tips of the two sets of leaves dipped in water and roots and shoots developed only in the submerged notches (Fig. 1).

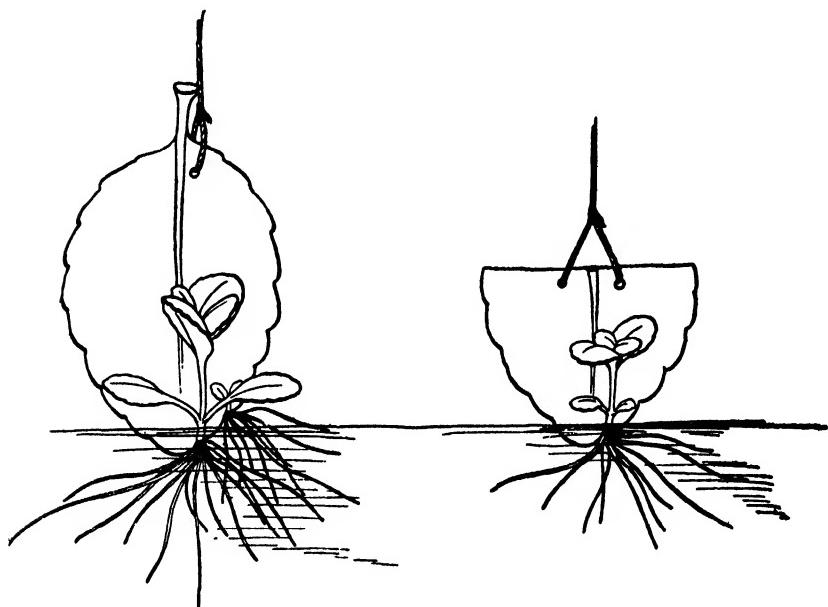


FIG. 2. Shoot and root formation in whole leaf and in sister leaf (from which the basal part is cut off), both dipping with their apices in water. Duration of experiment 34 days. After nature.

TABLE III.  
*Duration of Experiment 33 Days.*

	Dry weight of		
	Leaves.	Shoots.	Roots.
Set I (six reduced leaves).....	0.794	0.156 (11)	0.043
" II (" whole " ).....	2.127	0.343 (19)	0.116
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$ .....	0.37	0.45	0.37

In the next experiment one set of six leaves remained intact (Set II) while more than half of the basal part of the set of sister leaves (Set I) was cut off (Fig. 2). The tips of the leaves dipped into water. Again the ratio of the mass of shoots and roots regenerated was, within the limits of the accuracy of the experiments, the same as the ratio of the masses of the two sets of leaves. It was 0.37 for the leaves and exactly the same number for the roots, while it was slightly larger, 0.45, for the shoots (Table III).

In the next set, the leaves were suspended sidewise, their lower edges dipping in water (Fig. 3). In both sets of leaves the upper edge was cut off; in one set the lower edge with the exception of one

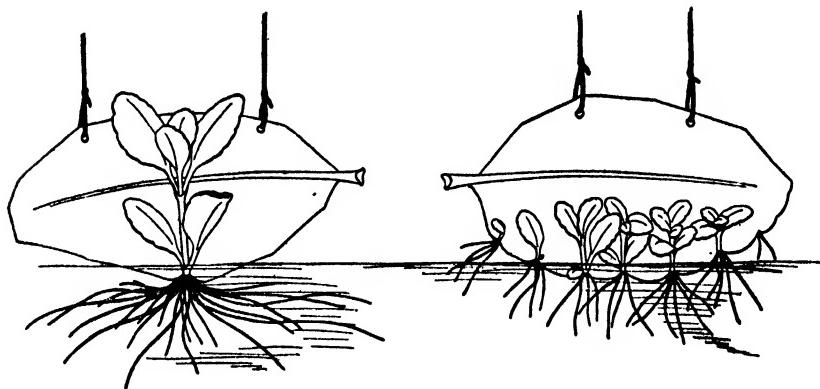


FIG. 3. Leaves suspended sidewise. The upper edge is cut off in both leaves. In leaf to left all notches except one removed, in leaf to right all notches on the lower side preserved. Duration of experiment 34 days. After nature.

or two notches was cut off (Set I), while in the other set (Set II) all the notches of the lower edge were left intact. This was done to make the number of shoots produced by both sets unequal and to show that the general law enunciated is independent of the number of shoots produced. Thus, as Table IV shows, the leaves of Set I produced six and the leaves of Set II produced sixteen shoots, yet the mass of the two sets of shoots was almost the same, though it is not impossible that when more shoots develop in one leaf the available material furnished by the leaf can be utilized more fully than when all the material goes into one shoot.

On the basis of these and of the writer's earlier experiments we can enunciate the following law.

Equal masses of sister leaves of *Bryophyllum calycinum* produce in equal time, under equal conditions of temperature, moisture, illumination, and aeration, approximately equal masses of roots and shoots, regardless of the number of shoots or roots formed (except that a moderate number of shoots may possibly permit a more complete utilization of the material furnished by the leaf than if only one shoot is formed). When the masses of two sister leaves are unequal, the masses of shoots and roots produced by them are directly proportional to the masses of the leaves.

TABLE IV.

*Duration of Experiment 33 Days.*

	Dry weight of		
	Leaves.	Shoots.	Roots.
	gm.	gm.	gm.
Set I. Five leaves with one or two notches....	1.810	0.248 (6)	0.106
" II. " sister leaves with more " ....	1.778	0.270 (16)	0.121
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$ .....	1.02	0.92	0.88

*II. Ratio of the Mass of Shoots to the Mass of Roots Produced by a Leaf Dipped in Water.*

When we dip a leaf with part of its edge into water, shoots and roots form as a rule only in the wetted part of the leaf. It was of importance to determine the ratio of dry weight of roots to the dry weight of shoots produced in such cases. In the examples already quoted this ratio was never less than one-third and never more than one-half. The average taken from a large number of experiments gave the ratio of the dry weight of roots to that of shoots under such conditions as 0.42. This figure will be used in the experiments of the next chapter.

### *III. The Inhibitory Influence of the Stem on Shoot Production in the Leaf of *Bryophyllum calycinum*.*

Only the leaf isolated from the stem is capable of forming shoots; it suffices as a rule to leave a piece of stem connected with the leaf to cause retardation or inhibition of the growth of the dormant buds in the notches of a leaf.<sup>2</sup> There are two possible reasons for the inhibitory action of the stem on the shoot and root formation in the leaf. The material available for root and shoot formation in the leaf either flows naturally into the stem and hence the buds in the notches of the leaf cannot grow out on account of lack of material for growth;<sup>3</sup> or second, the stem sends into the leaf a substance preventing the growth of the notches in spite of the fact that the material needed for the growth of the dormant buds in the notches of the leaf is available.

Only quantitative experiments allow us to decide between the two possibilities. If the assumption is correct that the leaf normally sends the material which can be utilized for the growth of shoots and roots into the stem, the stem in connection with a leaf should gain in weight and this gain should be equal to the mass of shoots and roots the same leaf would produce when separated from the stem. If the other possibility is correct, and if we are dealing with the effect of inhibitory substances sent into the leaf, no such increase in the dry weight of the stem need occur. My experiments give a clear answer in favor of the first possibility; namely, that the inhibitory effect of the stem upon the shoot and root production in the leaf is due to the fact that the material in the leaf which could be utilized for shoot and root formation flows normally into the stem, as long as the leaf is connected with the stem and the sap flow is not interrupted; while when the leaf is separated this material becomes available for the growth of shoots and roots in the leaf, and the inhibition ceases. Of course, we cannot determine directly which mass of shoots and roots the inhibited leaf would have produced if

<sup>2</sup> This inhibition cannot be permanent since before the falling off of the leaf the sap flow between leaf and stem will cease and this will have the same effect as the cutting off of the leaf.

<sup>3</sup> Loeb, J., *Ann. Inst. Pasteur*, 1918, xxxii, 1.

its regeneration had not been inhibited by the stem, but we can determine this quantity indirectly, by measuring the quantity of shoots and roots produced by the sister leaf detached from the stem, since we know that two sister leaves of equal size produce equal masses of shoots and roots in equal time and under equal conditions.

Our method of procedure was as follows. Pieces of stem possessing one node with two healthy leaves of equal size were cut out (Fig. 4). The stem was divided lengthwise as accurately as possible into two equal pieces,  $b$  and  $b_1$ , by a cut between the base of the petioles of the two leaves, so that each leaf was connected with one-half piece of stem of equal mass. One leaf ( $a_1$ ) remained in connection with its piece of half stem ( $b_1$ ), while the other leaf ( $a$ ) was detached from its piece ( $b$ ). The dry weight of this detached piece of half stem  $b$  was determined immediately at the beginning of the experiment.

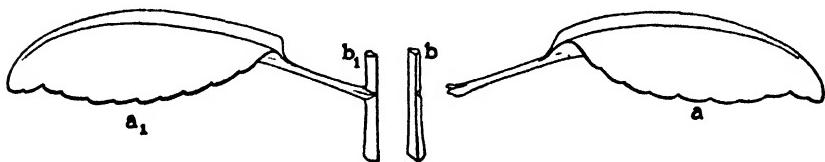


FIG. 4. Diagram to illustrate experiments in which the stem is cut lengthwise.

At the end of the experiment the other piece of half stem  $b_1$  was detached from its leaf and its dry weight was determined. By comparing the dry weight of  $b_1$  with that of  $b$  (detached at the beginning of the experiment) it was possible to ascertain how much material the piece  $b_1$  had received from the leaf during the time the experiment lasted. Both leaves, the one detached from the stem as well as the one left in connection with the stem, were suspended in a moist aquarium, the apex of each leaf dipping in water. The detached leaves formed shoots and roots very rapidly, while the leaves in connection with their half pieces of stem formed practically no shoots and roots.<sup>4</sup>

<sup>4</sup> The inhibitory power of a piece of stem on the shoot formation in the leaf increased within certain limits with the mass of the stem, but not in direct proportion. It was also very obvious that equal masses of stem suppressed the

We selected for each experiment twelve pairs of sister leaves and care was taken that the leaves were healthy, not too large, and of equal size. We made sure that the fresh weight of each set of leaves with their piece of half stem was approximately like that of the set of sister leaves (Table V).

The difference in one set is not more than 3 per cent and is equalized by the fact that six sets of different experiments were made. We proceeded then as follows. The dry weight of  $b$  (Fig. 4) was ascertained immediately. Then the leaf  $a$  (detached from its stem) and the leaf  $a_1$  (in connection with its piece of half stem) were suspended in the same aquarium dipping with their apices into water. In the leaves  $a$  (without stems), roots and shoots formed soon, while none, or practically none, formed in leaves  $a_1$ .

TABLE V.

No. of experiment.	Fresh weight of	
	$a + b$	$a_1 + b_1$
	gm.	gm.
1	28.240	29.103
2	36.625	37.175
3	40.930	39.640

After several weeks the shoots were cut off, their dry weight was determined, and the piece of stem  $b_1$  was also detached from its leaf and its dry weight determined. Let  $a$  be the dry weight of shoots and roots formed by the leaves detached from their stem; let  $a_1$  be the dry weight of shoots and roots formed by the leaves or in the axil of the leaves connected with their stem; let  $b$  be the dry weight

shoot formation the more completely the smaller the leaf. When the leaf is large it furnishes more material than the cells of a small piece of stem can absorb. It is also of interest that the inhibitory power of a piece of stem on shoot formation in a leaf is smaller in an old leaf than in a young leaf nearer the apex. This influence of age of the leaf is probably connected with the fact that in the oldest leaf the flow of sap into the leaf is more or less incomplete; this interruption of sap flow may possibly be the first step in the histological changes at the base of the leaf which cause it to fall off. In our experiments conditions were selected in such a way as to make the inhibition of shoot and root formation in Leaf  $a_1$  practically complete.

of the piece of half stem at the beginning,  $b_1$  the dry weight of the piece of half stem at the end; then we should expect the following relation to hold (within the limits of accuracy of the experiments).

$$a - a_1 = b_1 - b$$

Six sets of experiments (each with twelve pairs of leaves) were made in which the dry weights of the shoots and of the pieces of stem were determined. The stems were either 2, 4, or 8 cm. long, and with this length of stem the formation of shoots on the pieces connected with the stem was almost completely suppressed. The weights of the roots were not determined but we know that on the average the dry weight of the roots formed by the leaves under the

TABLE VI.

No. of experiment.	Duration of experiment. days	$a$	$a_1 + b_1$	$b$	$a_1 + b_1 - b$
		gm.	gm.	gm.	gm.
1	28	0.141	0.384	0.176	0.208
2	28	0.172	1.1095	0.825	0.284
3	28	0.308	0.7348	0.3846	0.350
4	29	0.216	0.496	0.159	0.337
5	29	0.166	1.146	0.829	0.317
6	29	0.267	0.759	0.364	0.395
Total.....		1.270			1.891

conditions of our experiments is 42 per cent of the dry weight of the shoots formed. Hence we must add this value in our final results to  $a$  (but not to  $a_1$ , since practically no shoots or roots were formed in the leaves connected with the stems). Table VI gives the dry weight determinations for the six sets of experiments. The dry weight of the shoots produced by all the detached leaves in the six sets of experiments is 1.270 gm. To this value must be added the dry weight of the roots produced which amounts to 42 per cent of the dry weights of shoots; namely, 0.533 gm. The value for  $a$  then is  $1.270 + 0.533 = 1.803$  gm.

The value for  $a_1 + b_1 - b$  was equal to 1.891 gm. Practically no shoots or roots were produced by these leaves connected with their stems, so that no correction for roots is necessary. Hence the value

1.891 represents the gain in mass of the stem. We therefore find that  $a = 1.803$ , and that  $a_1 + b_1 - b = 1.891$ . The ratio of  $\frac{a}{a_1 + b_1 - b}$  should be = 1. In our experiments it is = 0.95, which is as near the theoretical value as the accuracy of our experiments permits.

We, therefore, can state: When a leaf is connected with a piece of stem the stem gains in weight and this gain equals the weight of roots and of shoots formed by the sister leaves detached from the stem. The inhibitory effect of the stem upon shoot and root formation by the leaf is therefore adequately explained by these figures as being due to the fact that the material available for growth normally flows from the leaf into the stem.

#### SUMMARY.

1. Equal masses of sister leaves of *Bryophyllum calycinum* produce equal masses of shoots and roots in equal time and under equal conditions.
2. The mass of shoots and roots produced by different masses of sister leaves in equal time and under equal conditions is approximately in direct proportion to the masses of the leaves.
3. When a piece of stem inhibits the production of shoots and roots in a leaf of *Bryophyllum* connected with it, the stem gains in mass and this gain in mass equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem.
4. This suggests that the inhibitory influence of the stem upon the formation of shoots and roots in the leaf is due to the fact that the material available for this process naturally flows into the stem.



## A DEVICE FOR REGULATING THE TEMPERATURE OF INCUBATORS EITHER ABOVE OR BELOW ROOM TEMPERATURE.

By JOHN H. NORTHRUP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 23, 1920.)

It is frequently desirable in studying the effect of temperature on living organisms to have a convenient means of regulating the temperature of an incubator or series of incubators within the range of from 5-40°C. For this purpose it is essential to have a regulating device which is reliable over long periods of time with a variation of 0.2-0.3°. As far as the author is aware no convenient method has been described for this purpose.

The device described in this paper has been in use continually for several years and has proved reliable and accurate. It consists essentially in regulating the flow of water through the jacket of a double-walled incubator. This is accomplished, as shown in Fig. 1, by causing a relay to direct a stream of water either through the incubator or to waste as required by the temperature changes. This is brought about by means of a wire D soldered to the armature of the relay and attached at the other end to a glass pipette at the end of a vertical rubber tube. The current necessary to move this wire and pipette is so small that it is unnecessary to use a secondary circuit. The relay is actuated directly by the same circuit which goes through the regulator. With a relay of 150 to 200 ohms resistance, a potential difference of about 1 volt is necessary. This may conveniently be obtained from the ordinary lighting circuit by the use of lamps as shown in Fig. 1. The regulator may be any convenient type, either mercury-toluene or bi-metallic. It is placed in the regular position in the incubator.

The adjustment for temperatures higher than that of the room is as follows: Hot water is allowed to run in a slow stream from the

pipette A which is so adjusted that, when the circuit closes and the armature of the relay is pulled over, the water flows into a funnel B

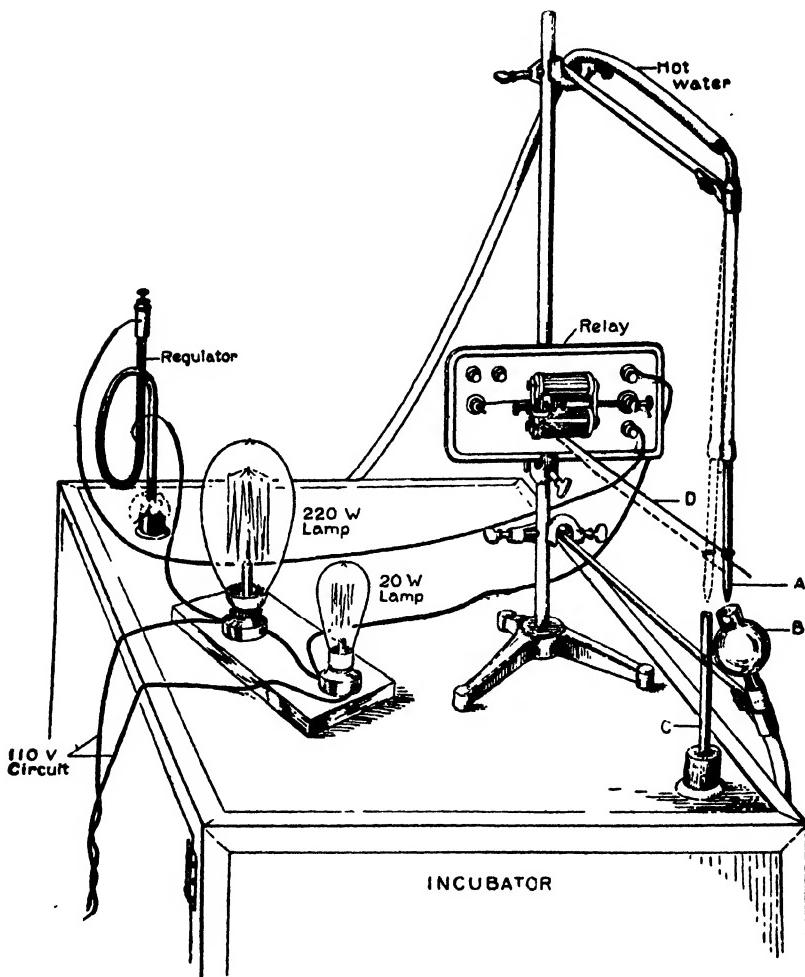


FIG. 1. Device for regulating the temperature of incubators.

placed near the opening C in the incubator jacket and thence to waste. When the circuit is broken the water flows into the incubator and so raises its temperature. The overflow from the incubator

runs off from the top of the water gauge on the side of the incubator (not shown in Fig. 1).

The adjustment for temperatures below room temperature is identical except that cold water is run through A and the relay so adjusted that the water runs into the incubator when the circuit is closed and to waste when the circuit is open. Temperatures to 8 or 10°C. may easily be maintained by the use of ice water.

When temperatures within the range of variation of the room are desired, a slow, continuous stream of cold water is run through the incubator and warm water is run through the pipette connected with the regulating device.



## CONCERNING THE HEREDITARY ADAPTATION OF ORGANISMS TO HIGHER TEMPERATURE.

By JOHN H. NORTHRUP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 26, 1920.)

Since the time of Lamarck the theory of the adaptation of organisms to their environment and the inheritance of these adaptations has been a hotly debated question. The original theory considered that the organism responded to changes in environment in such a way as to become better fitted to it, and that these changes then became hereditary. In this form the theory is probably no longer accepted by the majority of students. There is also little doubt that structural adaptations of the individual organisms are not inheritable. A general discussion of this question is out of place here, and the reader is referred to Loeb<sup>1</sup> and Conklin.<sup>2</sup> It may be pointed out, however, that according to the accepted theory of Weismann in regard to the continuity of the germ plasm, it seems *a priori* improbable that any change in the individual could affect succeeding generations.

Morgan and his coworkers<sup>3</sup> have shown that structural changes are continually arising in many forms, and that these changes or mutations are inherited according to Mendel's law. There seems no reason to suppose that physiological changes might not arise in the same way. If these physiological changes were such that the organism became better fitted to a new environment, we might expect to find adaptation under some conditions, although it would not be the direct result of the changed environment. Tower<sup>4</sup> has reported

<sup>1</sup> Loeb, J., *The organism as a whole from a physicochemical viewpoint*, New York, 1916.

<sup>2</sup> Conklin, E. G., *Heredity and environment in the development of men*, Princeton, 1915.

<sup>3</sup> Morgan, T. H., Sturtevant, A. H., Muller, H. J., and Bridges, C. B., *The mechanism of Mendelian heredity*, New York, 1915.

<sup>4</sup> Tower, W. L., *Biol. Bull.*, 1917, xxxiii, 229.

experiments which apparently showed some such result. In the bacteria and other unicellular organisms, there is no doubt that cultures may be adapted to very marked changes both in temperature and concentration of toxic substances. This is, however, analogous to the adaptation of an individual multicellular organism, and cannot be considered hereditary in the sense in which the word is used in regard to higher organisms. The individual cells of a bacterial culture bear the same relation to each other as the somatic cells of a single multicellular organism, and are not at all analogous to successive generations of individuals of multicellular organisms.

The experiments reported in this paper were made with aseptic cultures of *Drosophila*. These are especially favorable for such a study for the following reasons. (1) If kept free from microorganisms the results of any experiments made with them become quite regular. (2) They have a very short generation time, about 7 days at 30°. (3) Loeb and Wasteneys<sup>5</sup> found that the individual *Drosophila* show the same marked adaptation to temperature as does *Fundulus*. In the case of the latter, Loeb and Wasteneys found that fish transferred suddenly from 10 to 35°C. died in the course of 1 to 2 hours, whereas fish transferred first to 27° for 2 or 3 days and then put at 35° were able to live indefinitely at this temperature. The results with *Drosophila* showed equally striking individual adaptations.<sup>6</sup> These experiments were partially repeated and confirmed in the course of the present work.

The relation of the rate of growth and of the duration of life of *Drosophila* to the temperature has been the subject of a previous paper from this laboratory.<sup>7</sup> It was found in that work that the insects developed normally up to a temperature of 32.5°C. Above this temperature the pupal stage was injured and no further development took place; but the larval and imago stages could live at a temperature several degrees higher. It was also found that increasing the temperature from 10 to 27.5°C. increased the rate of development of the larvæ and pupæ; but that between the temperatures of 27.5 and 32°C. the rate decreased again; i.e., the larvæ grow more slowly at

<sup>5</sup> Loeb, J., and Wasteneys, H., *J. Exp. Zool.*, 1912, xii, 543.

<sup>6</sup> Loeb, J., and Wasteneys, H., unpublished data.

<sup>7</sup> Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

either 25 or 30° than they do at 27.5°. This secondary decrease in the rate at temperatures above 27.5° was compared with a similar decrease in the rate of enzyme action, and was ascribed to a similar cause; namely, injury and subsequent slowing up of the growth processes.

In all these experiments the eggs whose development was studied were produced by imagos which had been raised at temperatures of from 15–20°C. If there was any hereditary adaptation to higher temperature it would be expected that flies which had developed near the upper temperature limit would be able to produce eggs slightly

TABLE I.

*Effect of Temperature at which Parent Generation Develops on Upper Temperature Limit for Development of the Succeeding Generation.*

Temperature at which parent imagos were raised. °C.	Temperature of development of succeeding (F <sub>1</sub> ) generation. °C.	Condition of succeeding (F <sub>1</sub> ) generation after days noted.			
		2 days.	5 days.	7 days.	15 days.
20	29	Eggs. <sup>3</sup>	Larvæ.	Pupæ.	Imagos.
32	29	"	"	"	"
20	32	"	"	"	"
32	32	"	No larvæ.	No larvæ.	No larvæ.
20	33	"	Larvæ.	Pupæ.	Pupæ dead.
32	33	"	No larvæ.	No larvæ.	No larvæ.

more resistant to temperature (*i.e.* able to develop at a slightly higher temperature) than flies which had developed at a lower temperature. In order to test this assumption, cultures of imagos which had developed at 20 and 32° respectively were placed in incubators at 29, 32, and 33°. The development of the eggs produced by these imagos was then followed. The results are summarized in Table I.

It will be seen that those imagos which had developed at 20° and were then transferred to a temperature of 29 or 32° were able to produce eggs capable of developing into imagos at that temperature. The eggs produced at 33°, however, do not develop beyond the pupal stage. The imagos which had developed at 32° are unable to produce eggs capable of development into imagos at temperatures higher than 29°. The effect of raising *Drosophila* at high temperatures,

therefore, is to lower the upper temperature limit for the development of the succeeding generation and not to elevate it as would be expected if the adaptation to temperature was hereditary.

It might be objected that the results shown in Table I are not due to any difference either in the eggs or imago but merely to the fact that, in the case of the imagos raised at 20°, the eggs which are to give rise to the succeeding generation pass through the early stages of development within the female while at the lower temperature and so escape injury; while in the case of the cultures kept continuously at 32° the early stages of the eggs must necessarily be passed at this temperature and the eggs are thereby injured. If this was the case, only those eggs produced by the 20° culture immediately after being transferred to 32° should develop, and the ones produced later should fail to develop. This, however, is not so. Imagines raised at 20° and transferred to 32° can produce eggs, capable of developing at this temperature, for a week or 10 days after having been transferred from the lower temperature.

It was found that imagos, raised and kept permanently at a temperature of 30°, are unable to produce eggs capable of development at this temperature. If, however, they are removed from the 30° incubator within a week after emerging from the pupæ and placed at a temperature of about 20°C. for 24 hours or longer, they become able to produce eggs capable of development at 30°C. when replaced at this temperature. Table II is a summary of an experiment illustrating this point. It is necessary to remove the imagos from the higher temperature within a week or 10 days after they have emerged from the pupæ. If they are left longer at the higher temperature, the injury becomes permanent and they are no longer able to produce eggs capable of development at any temperature.

It is therefore not possible to raise more than one generation of *Drosophila* at a temperature of 29° or over unless the culture is removed to a lower temperature for at least 24 hours every generation.<sup>8</sup> A culture has been continued at 30° by this method of intermittent

<sup>8</sup> This fact may seem surprising since the organism is a tropical form. The explanation is probably that the temperature even in the tropics does not stay continuously higher than 28 or 30° for more than a week or 10 days.

cooling for ten generations without any noticeable change in the upper temperature limit. A second culture was kept continuously at 28° for fifteen generations. In this case also there has been no noticeable change in the temperature limit; *i.e.*, the organisms are still unable to grow for more than one generation at a continuous temperature of 29° or over.

TABLE II.

*Effect of Placing Cultures, Raised at 30°, at 22°C.*

Days.

1. 50 to 100 imagos from normal aseptic culture generation No. 89 (raised at 20°) placed at 30°C.
2. Many eggs laid.
4. Larvæ developing. Parent imagos removed.
8. Imagos of the new generation ( $F_1$ ) placed at 22° and put back at 30° after varying time intervals as stated below.

Length of time at 30° C.	Time during which ( $F_1$ ) cultures were left at 22°C.				
	0.25 hr.	4 hrs.	24 hrs.	96 hrs.	200 hrs.
Stage of development of ( $F_2$ ) generation cultures after days at 30°C.					
days					
3	Eggs, but no larvæ.	Eggs, but no larvæ.	Both eggs and larvæ.	Both eggs and larvæ.	Both eggs and larvæ.
5	Eggs, but no larvæ.	Eggs, but no larvæ.	Both eggs and larvæ.	Both eggs and larvæ.	Both eggs and larvæ.
10	Eggs, but no larvæ.	Eggs, but no larvæ.	Pupæ.	Pupæ.	Pupæ.
15	Eggs, but no larvæ.	Eggs, but no larvæ.	Normal imagoes.	Normal imagoes.	Normal imagoes.

## EXPERIMENTAL.

*Temperature Control.*—The cultures were kept in water jacketed incubators regulated as described in another paper.<sup>9</sup>

*Food.*—All cultures were fed on a sterilized suspension of yeast in water. The excess water was absorbed by cotton added to the flask, as described below.

*Method of Transferring Cultures, Etc.*—The insects were kept in 1 liter flat bottom Florence flasks having a side tube fused on as in a Pasteur flask. This side tube was closed with a rubber tube and glass plug, and the neck of the flask plugged with cotton. About 25 cc. of a thick suspension of yeast in water were

<sup>9</sup> Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 309.

added to the flask, the excess water was absorbed by the addition of absorbent cotton, and the flask sterilized. It is important to add sufficient cotton to absorb the water as otherwise the insects stick to the side of the flask and are drowned. In order to transfer the culture, the flask is connected to one containing the insects by means of the side tubes, using the same technique as in handling a Pasteur flask. The flies are then shaken from one flask to the other through the connecting side tubes, the flasks disconnected, and the connecting tubes flamed and re-plugged. In this way the organisms can be handled with as little danger of infection as cultures of bacteria.

#### SUMMARY.

1. Imagos of *Drosophila* raised at temperatures of from 12–28.5°C. when placed at any temperature from 15–32.5°C. produce eggs which develop normally at these temperatures.
2. Imagos raised at temperatures of from 29–32.5° and then kept permanently within these temperatures produce eggs which do not develop.
3. Imagos raised at from 28.5–32.5°C. and then placed at temperatures of from 12–25°C. produce eggs which develop normally.
4. Imagos raised at from 28.5–32.5°C. placed at 15–25°C. for 24 hours or longer and then put back into a temperature of from 28.5–32.5°C., produce eggs which will develop at the latter temperature.
5. There is no evidence of any hereditary adaptation to higher temperatures.

## THE NATURE OF THE DIRECTIVE INFLUENCE OF GRAVITY ON THE ARRANGEMENT OF ORGANS IN REGENERATION.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 16, 1920.)

### I.

In preceding papers it has been shown that gravity has a directive influence on the arrangement of certain organs in the regeneration of *Bryophyllum calycinum*. Thus, when an excised piece of stem of *Bryophyllum* is suspended horizontally in moist air, roots will grow abundantly on the lower side of the stem but not on the upper side, with the exception of the basal end where the roots will grow on both sides. In order to get abundant root formation it is necessary to preserve one of the apical leaves on such a stem since the leaf furnishes most of the material for the growth of the roots.<sup>1</sup>

A second case in which this directive influence shows itself is the leaf of the same plant. When we suspend an isolated leaf of *Bryophyllum* (in moist air) sidewise, in a vertical plane, roots and shoots will grow abundantly in the notches of the lower side, but less abundantly and frequently not at all in the notches of the upper side of the leaf (Fig. 1, upper row). This phenomenon was explained by the writer in the following way. Gravity causes a slightly greater collection of sap on the lower side of the organs mentioned and this causes the dormant buds for root formation to grow out a little earlier or more quickly on the lower than on the upper side of the stem or the leaf. It is a general rule in the phenomena of regeneration in *Bryophyllum* that organs which grow out first or more quickly attract for some reason the flow of sap (possibly by modifying the direction of the sap flow) and thereby inhibit or retard the growth of similar organs in other places, and this inhibitory factor is added to the influence of

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 687.

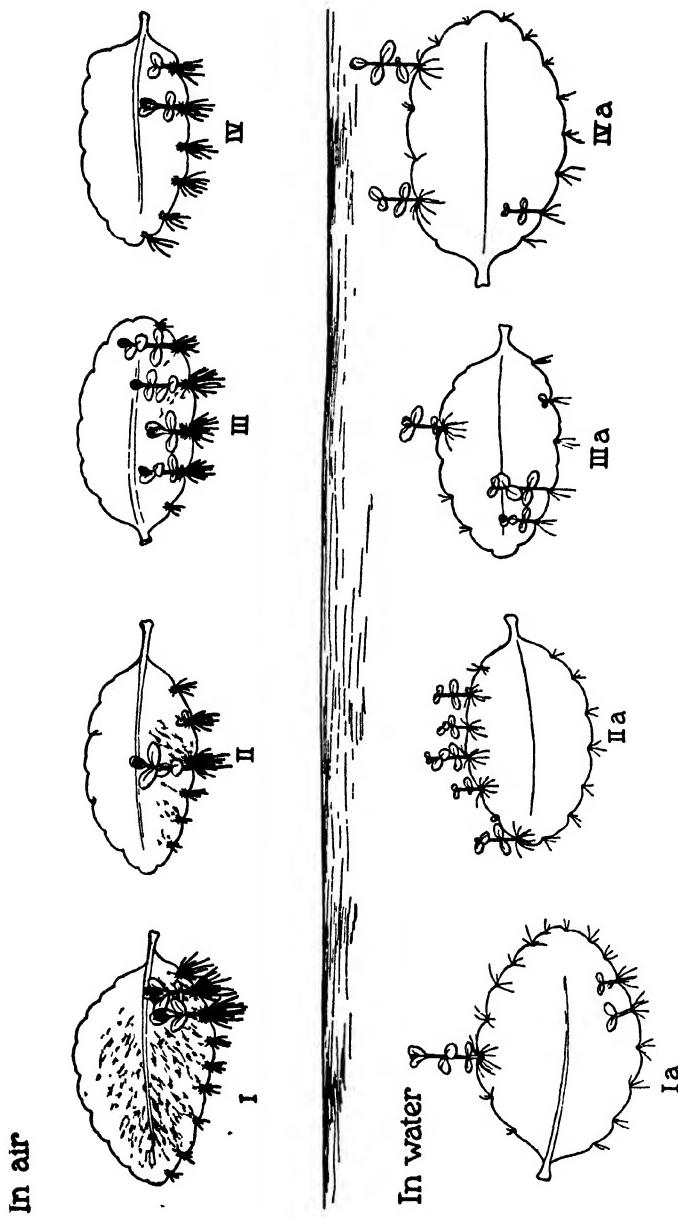


FIG. 1. Upper row, leaves suspended in air, lower row, leaves suspended in water. In moist air (upper row) roots and shoots are found only in the notches of the lower side of the leaves, while in water this directive influence of gravity does not exist, inasmuch as roots and shoots are formed on both the upper and the lower sides of each leaf. Only the leaves in the upper row show the red pigment indicated by stippling. Duration of experiment, Oct. 26 to Dec. 15, 1919.

gravity upon the distribution of sap. It is, therefore, not gravity alone which determines the directive effect in these cases but gravity in combination with the modification of the sap flow towards a growing organ.<sup>1</sup>

This reasoning is supported by the following facts. When we prevent the growth of roots and shoots on the lower side of the leaf by cutting off the dormant buds contained in the notches of the lower side of a leaf of *Bryophyllum* suspended vertically and sidewise, roots and shoots will now develop as abundantly on the upper side as they otherwise would have developed on the lower side; the only difference being that the roots begin to appear on the upper side slightly later than they would have appeared on the lower side.<sup>1</sup>

The same proof can be furnished for the formation of roots on the lower side of a horizontally placed stem. When we cut off the lower half of such a stem the roots will now form abundantly on the upper side of the stem.<sup>1</sup>

The fact that some sap collects on the lower side of a piece of stem suspended horizontally or on the lower side of an isolated leaf suspended sidewise in a vertical plane may be compared to the behavior of edematous liquid in animals which also follows gravity. It is possible that only that part of the sap of an isolated organ is thus affected by gravity which is not in active circulation in the vessels. The circulation in the vessels will cause an abundant collection of material at the ends of a piece of stem around the whole circumference of the piece and this may account for the fact that at the basal end of an excised piece of stem suspended horizontally in air roots develop on both the lower and upper side in such abundance that the slight quantity of sap collected through the influence of gravity on the lower side of the base becomes a negligible factor.

The real share of gravity in the directive influence on root and shoot formation is, therefore, a modest one since it needs consist only in a slightly greater collection of sap on the lower side of an organ, just sufficient to accelerate the growth of roots on the lower side.

It occurred to the writer that this conception of the directive influence of gravity on root formation might be tested in still another way; namely, by suspending leaves or pieces of stem under water. In this case the influence of gravity on root formation should dis-

appear or be greatly diminished, since the influence of the water furnished from the outside might tend to make the influence of the slight quantity of sap collected on the lower side through the influence of gravity a negligible factor.

When we suspend pieces of stem of *Bryophyllum* horizontally under water (instead of in air) roots no longer develop on the lower side only but on the upper side as well (Figs. 2 and 3). The sap will in this case collect also in somewhat greater abundance on the lower side of the stem, but this will cause no retardation of growth on the

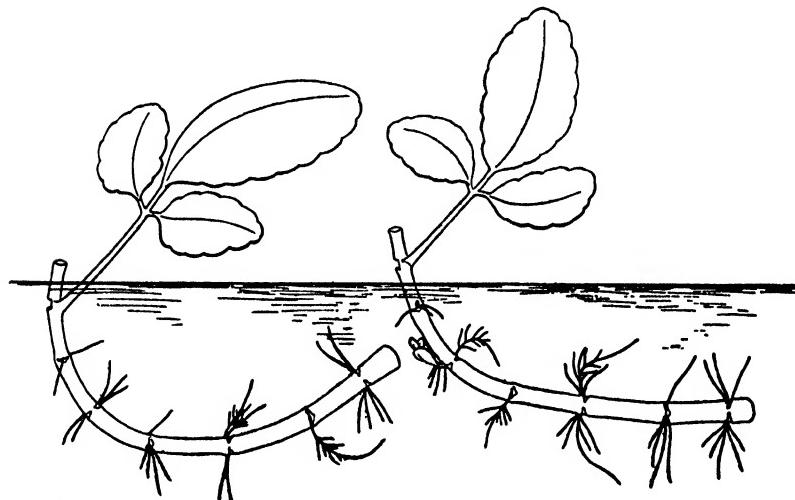


FIG. 2. Stems suspended horizontally under water. Roots form no longer exclusively on the lower side of the stems as they would have done had the stems been suspended in air, but on both the upper and lower side of the stem. The stems were originally straight and the curvature is due to geotropism; i.e., greater growth on the lower side.

upper side on account of the abundance of water on the upper side of the stem. This is also true for leaves. When we suspend leaves vertically and sidewise under water the roots and shoots will develop equally fast on the upper and lower sides of the leaf and the influence of gravity will disappear (Fig. 1, lower row). In both cases it is necessary to suspend the organs near the surface of the water so that their oxygen supply will not suffer too much.

We can finally submit our idea concerning the nature of the directive effect of gravity upon the arrangement of organs to a quantitative test. When an organ (*e.g.* a leaf suspended vertically and sidewise

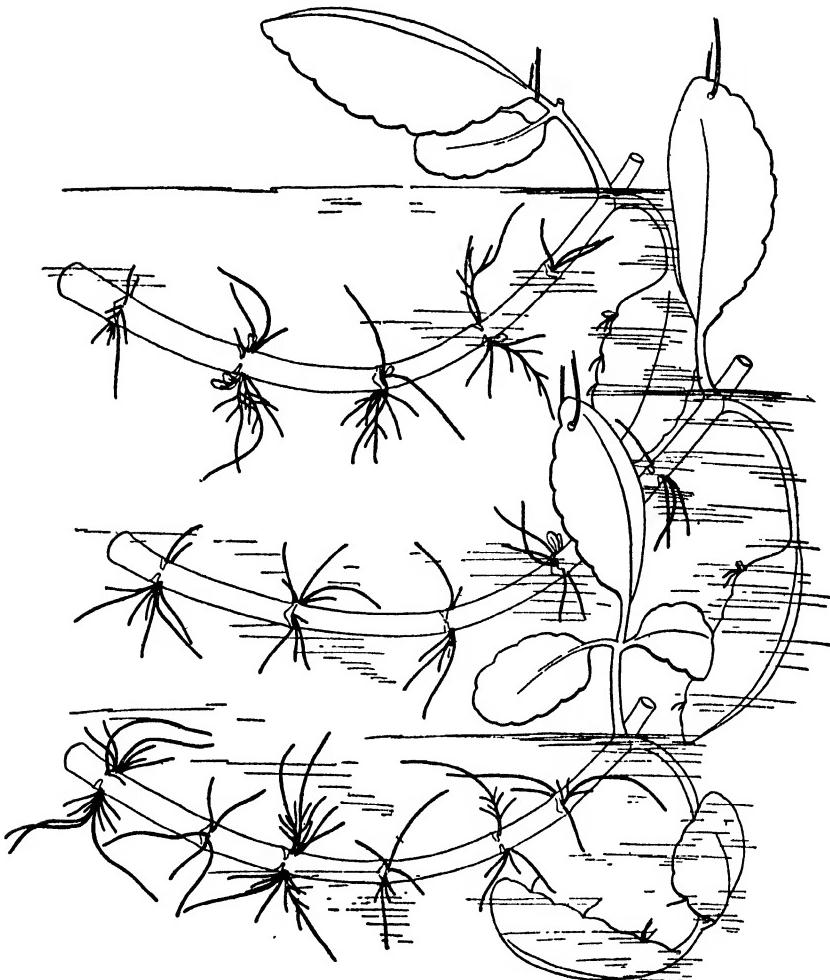


FIG. 3. Same as Fig. 2. Duration of experiment, Oct. 13 to Nov. 1, 1919.

in moist air) forms roots or shoots exclusively on the lower side, the material for these new organs must be partly withdrawn from the upper side of the organ and we must be able to prove that the dry

weight of the lower half of such an organ is always greater than the dry weight of the upper half; while when an organ forms shoots and roots on both the upper and lower side (*e.g.* a leaf suspended vertically and sidewise in water) no such difference in the dry weight of the upper and lower half should be expected. Determinations made by the writer confirm this expectation.

These experiments were carried on in the following way. A number of leaves were suspended vertically and sidewise in an aquarium

TABLE I.

*Dry Weight of Upper and Lower Halves of Leaves Suspended Vertically and Sidewise in Air. Roots and Shoots on Lower Side Only.*

Duration of experiment. <i>days</i>		Dry weight. <i>gm.</i>	Ratio
			<i>dry weight upper halves. dry weight lower halves</i>
A. 22	Upper halves of six leaves.	0.620	0.815
	Lower " " " "	0.751	
B. 32	Upper " " " "	0.810	0.800
	Lower " " " "	1.011	
C. 36	Upper " " " "	0.570	0.888
	Lower " " " "	0.642	
D. 35	Upper " " eight "	0.582	0.855
	Lower " " " "	0.681	
E. 31	Upper " " five "	0.499	0.872
	Lower " " " "	0.572	

filled with moist air. The majority of these leaves formed roots in abundance on the lower side but not on the upper side. For this experiment leaves with perfect symmetry were selected. After about 4 or 5 weeks a number of such leaves were cut as accurately as possible along the middle rib and the fresh and dry weights of the upper and lower halves were determined. The dry weight of roots and tiny shoots formed on the lower side was included in the dry weight of the lower halves of the leaves. The lower halves of the leaves had, without exception, a higher dry weight than the upper halves (Table I).

As a control the same experiments were made with leaves suspended under water which had formed roots and shoots on both their upper and their under sides though there were fewer on the upper side. In this case there was no constant difference between

TABLE II.

*Controls, Dry Weight of Upper and Lower Halves of Leaves Suspended Vertically and Sidewise in Water. Roots and Shoots on Upper and Lower Sides.*

Duration of experi- ment.		Dry weight.	Ratio <u>dry weight upper halves.</u> <u>dry weight lower halves</u>
days		gm.	
A. 29	Upper halves of six leaves with ten shoots.	0.561	1.04
	Lower " " " " eleven "	0.538	
B. 32	Upper " " five " " four "	0.672	1.11
	Lower " " " " two "	0.606	

TABLE III.

*Dry Weight of Upper and Lower Halves of Leaves Suspended Vertically and Sidewise in Moist Air, Which Formed Some Shoots on Both Upper and Lower Sides.*

Duration of experi- ment.		Dry weight.	Ratio <u>dry weight upper halves.</u> <u>dry weight lower halves</u>
days		gm.	
35	Upper halves of four leaves.	0.365	0.990
	Lower " " " "	0.369	
31	Upper " " seven "	0.735	0.940
	Lower " " " "	0.783	

the dry weights of the upper and lower halves. The differences found were very slight and occurred in both directions (Table II).

The same was true for leaves raised in moist air, which formed some shoots on the upper side. The dry weights of the mass of the upper and lower halves did not differ (Table III).

## II.

The idea that a collection of sap occurs in the lower parts of a leaf suspended in a vertical plane can be demonstrated through the fact that leaves thus suspended in moist air form a reddish or purple pigment which has a tendency to collect in the lower parts of such a leaf. Fig. 4 shows two leaves with this pigment. The leaves had been suspended in moist air from April 17 to May 14, 1919. The

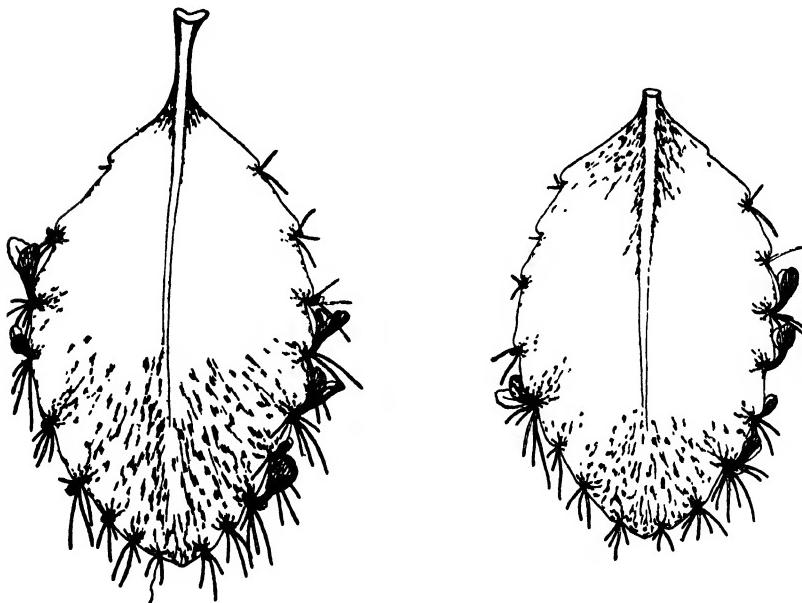


FIG. 4. Leaves suspended in moist air. Collection of reddish or purplish pigment in lower halves of the leaves, following the vessels. It is also obvious that the pigment collects more densely near each notch where a shoot or roots grow out. The leaves of the new shoots formed are full of pigment while the roots are free from it. Duration of experiment, Apr. 17 to May 14, 1919.

reddish or purple pigment is indicated in the drawing by black stippling. It is obvious that the pigment collects in the lowest parts of the leaf, that it follows the vessels, and that it also flows into the young leaves, thus supporting the view that a deflection of the sap flow towards the new shoots occurs in such a leaf. It does not,

however, collect in the new roots. A closer inspection of the leaves shows that the red pigment collects not only in the lower half of the leaves but also in those notches of the upper part of the leaf where roots or shoots are growing vigorously, thus supporting the view that the flow of sap is directed to rapidly growing organs. While a small amount of red pigment may possibly be visible in a leaf under normal conditions (especially along the edge of a young leaf), a noticeable amount is formed when the amount of water in the leaf is diminished.

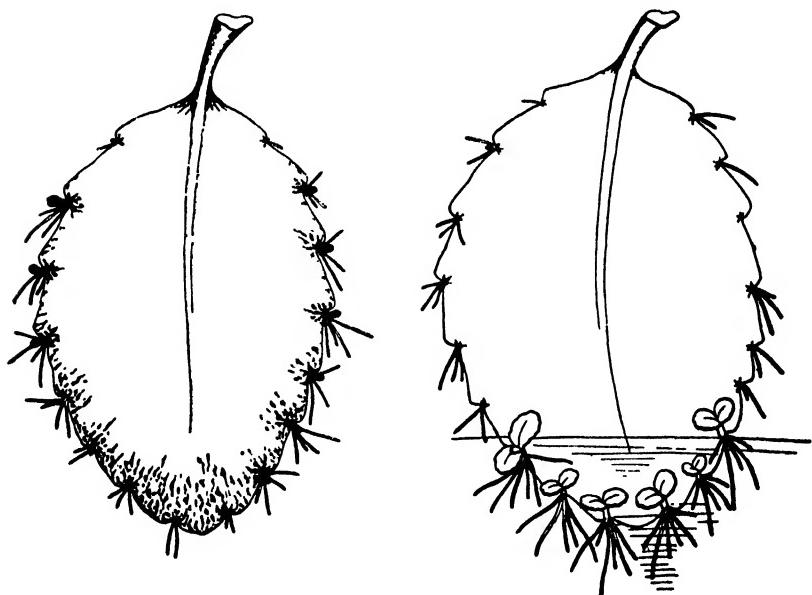


FIG. 5. Two sister leaves, one suspended in moist air, the other dipping with its apex into water. Only the former shows the pigment formation while the latter does not. This result is general and proves that the formation of the reddish pigment is favored by a diminution in the amount of water in the leaf since in the leaves suspended in moist air the ratio of dry to fresh weight is greater than in leaves which dip into water. Duration of experiment, Apr. 2 to Apr. 17, 1919.

This is shown by a comparison of the two sister leaves in Fig. 5, one of which (the one on the right) dipped with its apex into water while the other (the one to the left) was suspended in moist air. The ratio of dry weight to fresh weight of such leaves is always greater in the

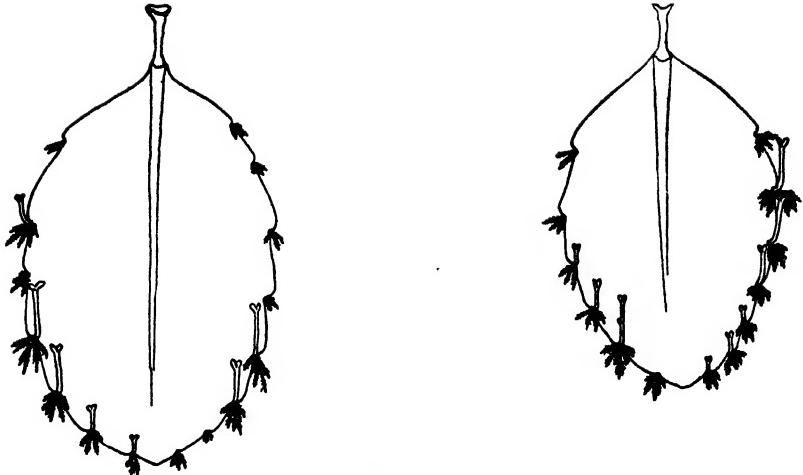


FIG. 6. Isolated leaves suspended in moist air but kept in dark. Although roots and shoots are formed no red pigment is noticeable. Duration of experiment, Apr. 20 to May 13, 1919.

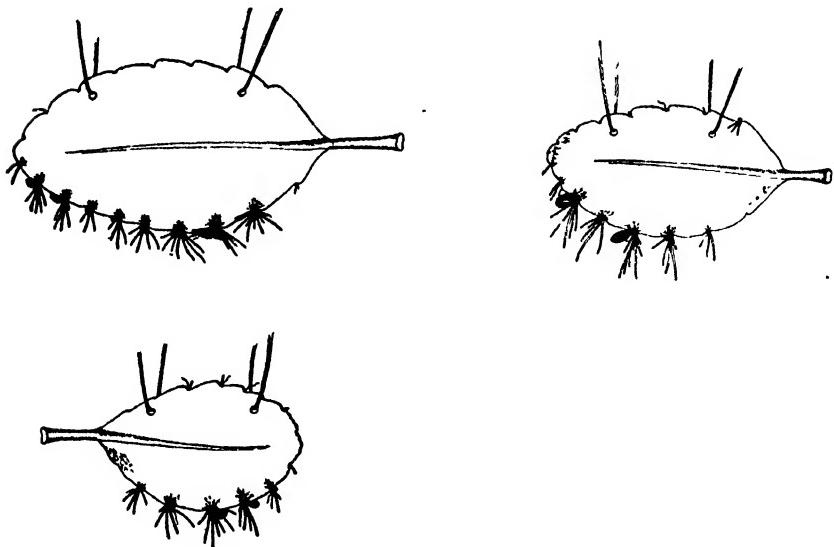


FIG. 7. Leaves suspended sidewise and in a vertical plane in moist air. The purple pigment collects near the notches on the lower side where roots and shoots develop. The young leaves are full of this purplish pigment. It collects also on the upper side of the middle rib while the lower side of the middle rib is free from pigment. In the petiole pigment exists on both the lower and upper side.

leaves suspended in air than in water. The leaf dipped into water (*i.e.* the leaf with a normal ratio of dry to fresh weight) formed no noticeable quantity of pigment while the other leaf with less water formed a considerable quantity. This difference is constant. A second factor necessary for the appearance of red pigment in the leaf is light. When leaves are suspended in moist air but kept in the dark they form no noticeable amount of red or purple pigment (Fig. 6) though they form roots and shoots.

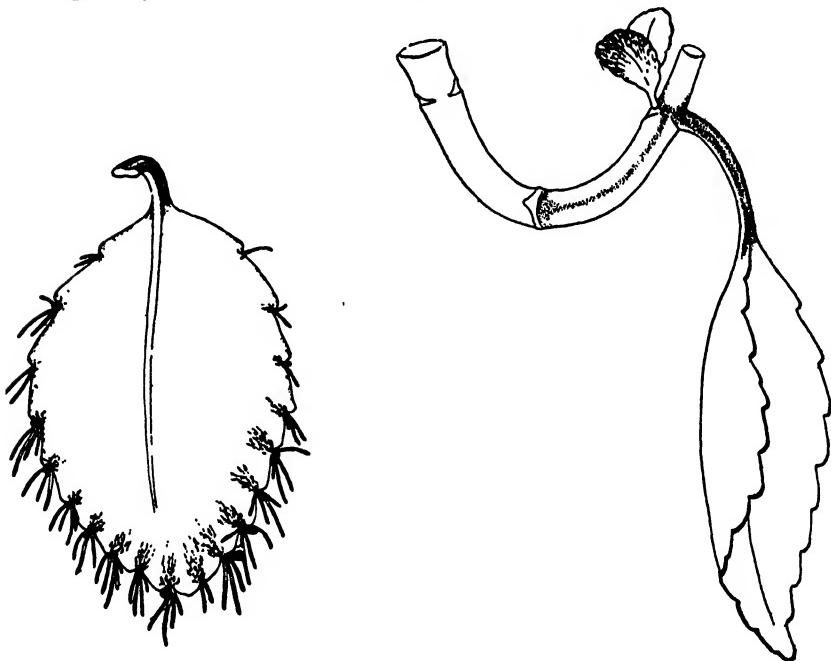


FIG. 8. Two sister leaves, one (to the right) connected with a piece of stem, the other (to the left) detached from the stem. Though both leaves are suspended in moist air only the detached (left) leaf is full of pigment, while the leaf in connection with stem is free from the purple pigment, which collects in the stem and in the leaves of the bud formed by the stem.

The most important fact for our problem is the distribution of sap in leaves suspended sidewise and in a vertical plane in moist air. In such leaves a collection of sap is found in the vessels close to the notches of the lower side of the leaves where the roots and shoots develop (Fig. 7).

We have stated in a preceding paper<sup>2</sup> that the sap from a leaf flows normally into the stem and that this is the reason of the inhibitory or retarding influence of the stem upon root and shoot formation in a leaf of *Bryophyllum*. This idea is supported by the fact that when a piece of stem is left in connection with a leaf suspended in moist air no collection of red or purplish pigment occurs in the leaf but that

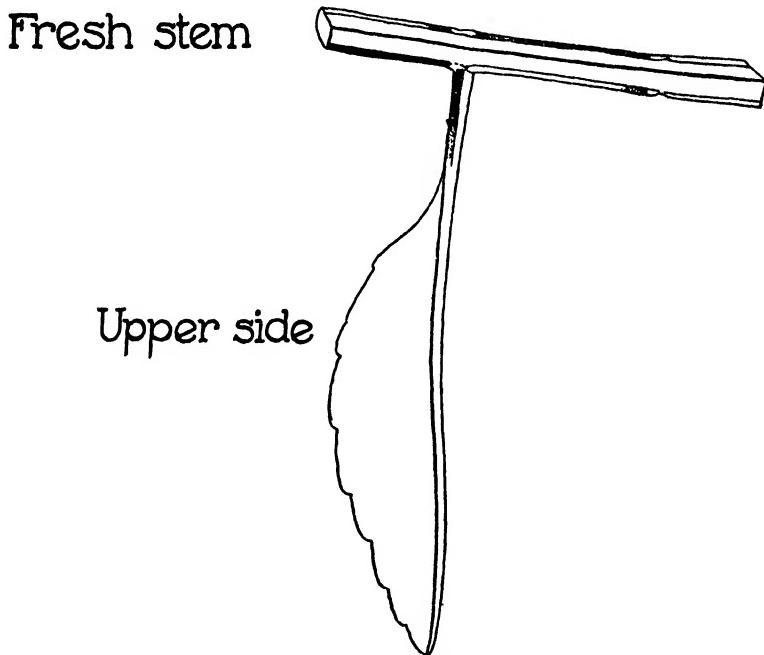


FIG. 9. Showing the distribution of the purple pigment in the stem.

it is found in the petiole, in the stem, and in the new shoots which are formed by the stem. This is illustrated by Fig. 8. The leaf on the right was left in connection with a piece of stem, while the sister leaf (to the left in Fig. 8) was detached from the stem. The latter shows a collection of the pigment in the vessels near each notch from which roots and shoots develop and the new shoots are full of the pigment. The sister leaf in connection with a piece of stem (to the right in

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 297.

Fig. 8) has no pigment in its notches, but the pigment is visible in the stem and especially in the small leaves formed on the upper side of the stem. It is needless to say that while only a few drawings are given the experiments were made on a large number of specimens, all yielding the same result. Fig. 9 shows the distribution of the pigment in the cortical layer of a piece of stem connected with a leaf.

While the purple pigment is a convenient indicator for the distribution of sap, it is not the cause of the growth of roots and shoots in a leaf. This is obvious from the fact that growth of roots and shoots takes place in isolated leaves when kept in the dark or when dipping in water, although no noticeable formation of the purple pigment takes place under these conditions.

#### SUMMARY AND CONCLUSIONS.

1. When leaves of *Bryophyllum calycinum* are suspended in moist air in a vertical plane and sidewise, roots and shoots are formed exclusively or predominate in the notches on the lower side of the leaves. When pieces of stems of the same plant are suspended horizontally in moist air, roots develop on the lower side of the stem, with the exception of the extreme basal end where they may develop on both sides.
2. The writer has suggested in a preceding paper that this directive influence of gravity on the arrangement of the regenerating organs may be due to the combination of two factors. The first factor is gravity, which causes a slightly greater collection of sap on the lower side of these organs, and as a consequence roots develop a little more quickly on the lower than on the upper side. The second factor is of an inhibitory character inasmuch as quite generally organs which grow out first, or which grow quickly, have a tendency to retard or inhibit the growth of similar organs in other places.
3. The writer was able to prove the action of this inhibitory factor by cutting off the lower edges of leaves suspended sidewise in a vertical plane or the lower halves of stems suspended in a horizontal plane (in moist air). In this case roots developed as abundantly on the upper side of these organs as they otherwise would have developed on the lower side.

4. It was, however, still necessary to prove the idea that gravity causes sap to collect in larger quantity in the lower parts of organs. This gap is filled by the present paper in which it is shown, first, that in the leaves suspended in moist air a red pigment is formed which has a tendency to collect gradually in the lowest parts of the leaf when the latter is suspended in a vertical plane. This red pigment serves as an indicator for the distribution of sap in the leaf and thus shows directly the tendency of the sap to collect in greater abundance on the lower edge of a leaf suspended in a vertical plane.

Second, it is shown that when leaves or stems of *Bryophyllum* are suspended, in the way described, under water instead of in moist air, roots develop on the upper side as well as on the lower side. The directive effect of gravity upon the arrangement of organs disappears in this case since the abundance of the outside water makes the effect of a slight difference in the distribution of sap between the upper and lower side a negligible factor.

Third, it is shown that the dry weight of the lower half of leaves suspended sidewise for several weeks in moist air in a vertical plane is greater than that of the upper half when roots and shoots are formed on the lower side only. This indicates that material from the upper half flows into the growing organs of the lower half. No such difference between upper and lower half of leaf is found when the leaves are suspended in the same way in water and roots and shoots are formed on both sides of the leaf.

5. It is shown that when a leaf connected with a piece of stem is suspended in moist air the red pigment goes into the stem instead of collecting in the lower part of the leaf, thus supporting the view expressed in a preceding paper that the inhibitory action of the stem on the root and shoot formation in a leaf of *Bryophyllum* is due to the fact that the material available in the leaf for organ formation is naturally sent into the stem.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, March 20, 1920, Vol. ii,  
No. 4, pp. 387-408.]

## ON THE CAUSE OF THE INFLUENCE OF IONS ON THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES. I.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 23, 1920.)

### I.

When we separate a watery solution from pure water by a collodion membrane water will diffuse into the solution and solute will diffuse out. We will call this diffusion free osmosis to distinguish it from osmosis due to an outside force; *e.g.*, electrical endosmose. The free diffusion of solute into pure water occurs at a rate proportional to the concentration of the solution<sup>1</sup> (with the possible exception of very low or very high concentrations) and need not occupy our interest in this paper. The diffusion of water into the solution has a different character when the solute is a non-electrolyte than when it is an electrolyte. When the solute is a non-electrolyte, the initial rate of diffusion of water into the solution is (within the limit of moderate concentrations) practically a linear function of the concentration of the solute, as it should be according to the law of van't Hoff. When the solution is an electrolyte, anomalies occur which are a characteristic function of the oppositely charged ions of the electrolyte and these anomalies were described for collodion membranes in a series of papers which have appeared recently.<sup>1, 2, 3, 4</sup> The anomalies seem to occur only in the lower concentrations of electrolytes, below  $m/8$  or less; above these values the osmosis seems to occur in a way similar to that observed in solutions of non-electrolytes, though this point

<sup>1</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 717.

<sup>2</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173.

<sup>3</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 255.

<sup>4</sup> Loeb, J., *Proc. Nat. Acad. Sc.*, 1919, v, 440.

needs further investigation. The present paper deals only with concentrations inside the anomalous range.

It was shown in the preceding papers that all the anomalies can be adequately described if we assume that the water in diffusing through the pores or interstices of the membrane is either positively or negatively charged and that the ions of the solution accelerate or retard the diffusion of the electrified water by their electrical charges. When we use collodion membranes which have been bathed for a short time in a 1 per cent solution of a protein (gelatin, casein, egg albumin, edestin, etc.) the effects of the two oppositely charged ions can be expressed in the following terms.

1. When we separate a neutral, alkaline, or faintly acid solution of an electrolyte with a monovalent or bivalent cation by a collodion membrane (treated with protein) from pure water, the latter diffuses into the solution as if its particles were positively charged and as if they were attracted by the anion and repelled by the cation of the solution with a force increasing with the valency of the ion and with a second constitutional quantity of the ion which we designated arbitrarily as the radius of the ion, but which needs another definition so as to include the strong effects of such monovalent ions as; e.g., the aluminate or oleate anion.

2. When we separate solutions of electrolytes with a concentration of hydrogen ions of about  $10^{-4}$  N or above, or with trivalent or tetravalent cations in sufficient concentration by a collodion membrane from pure water, water diffuses into the solution as if its particles were negatively charged and attracted by the cation and repelled by the anion of the electrolyte with a force increasing with the valency of the ion and with a second constitutional quantity of the ions still to be defined.

When we use collodion membranes *not* treated with protein one rule suffices to express all the phenomena; namely, water diffuses through the collodion membrane into the solution as if its particles were positively charged and as if it were attracted by the anion and repelled by the cation of the solution with a force increasing with the valency and a second quantity of the ion which is still to be defined.

What we designated in this statement as electrified particles of water is the watery phase or the mobile stratum of the electrical double layer (in the sense of Helmholtz) formed at the boundary of membrane and water or solution. The other stratum of this double layer, the membrane phase, must be considered as immobile in the experiments on osmosis. The influence of electrolytes on the double layer at the boundary of water and membrane will be assumed to be responsible for the phenomena of abnormal osmosis.

It follows from our previous publications that we must discriminate between two effects of electrolytes on the double layer. The one effect is the influence of certain ions on the sign of the electrification of the water or the solution phase of the double layer. Water in contact with a membrane is generally positively charged and only in the case of certain types of membranes, *e.g.* collodion membranes treated with proteins, can the sign of the charge be reversed by two kinds of ions; namely (*a*) hydrogen ions, or (*b*) simple (*i.e.* non-complex) cations whose valency is three or more.<sup>3</sup> All electrolytes which can lower the hydrogen ion concentration by a chemical action, *e.g.* alkalies, can restore the original positive electrification of the water stratum of the double layer. Some authors have drawn from this the conclusion that the OH ions act as directly as the H ions upon the sign of the electrification of the membrane (*e.g.* by transferring their negative charge to it). It seems to the writer that it is more in harmony with the facts to assume that the alkalies act merely by the diminution of the hydrogen ion concentration through neutralization of acid. The special effect of the two classes of positive ions—H ions and simple cations with a valency of three or above—on the sign of the electrification of the water stratum of the double layer will be discussed in another paper.

In addition to this *specific* influence of certain ions on the sign of the electrification of water at the boundary of the membrane there exists a second, more *general* effect of electrolytes on the *rate* of diffusion of water which was described in terms of electrostatic attraction and repulsion of the electrified stratum of water by the ions of the electrolyte in the previous papers, since this way of describing the results had the advantage of simplifying the presentation of the facts. It was not, however, intended to serve as a theoretical

basis for the explanation of the phenomena of abnormal osmosis. For this latter purpose we must express the facts in the following form. When we separate a solution of an electrolyte from pure water by a collodion membrane the oppositely charged ions of the electrolyte influence the initial velocity of diffusion of water through the membrane into the solution in an opposite sense; the ion with the opposite sign of charge from that of the electrified water (or the watery phase of the double layer) increasing the velocity, the ion with the same sign of charge as the watery phase of the double layer diminishing the velocity. The accelerating and retarding effects of ions were found to increase with the valency and with that other constitutional quantity which was designated as the radius of the ion but which requires further definition.

In passing we may remark that the relative retarding and accelerating effect of oppositely charged ions of an electrolyte on the rate of osmosis of water into the solution was not found to be the same for all concentrations of a solution.<sup>2</sup> At the lowest concentrations the effect of that ion usually (and possibly always) prevails which has the opposite sign of charge from that of the watery phase of the double layer, at a higher concentration the effect of that ion prevails which has the same sign of charge as the watery phase. Hence in the lower concentrations the accelerating effect of the electrolyte prevails over the retarding effect and for the higher concentrations the reverse is true. For a number of solutions, *e.g.* salts of monovalent cations, the turning point lies at a concentration of about M/256. The anomalous osmosis ceases at that concentration of the solution where the retarding and accelerating effects of the oppositely charged ions become equal. From then on the solutions of electrolytes seem to behave like those of non-electrolytes. This group of facts has been described in a preceding paper<sup>2</sup> and will not be discussed here.

In our experiments on free osmosis the collodion membrane was bounded on one side by pure water and on the other by the solution. When both sides of the membrane are bounded by identical solutions the rate of diffusion of water and of electrolyte in opposite directions is equal, and no change occurs. When, however, an external difference of potential is produced on the two sides of the

membrane, a transport of water or of liquid occurs through the membrane towards that electrode whose sign of charge is the opposite of that of the watery phase of the double layer in the pores or interstices of the membrane. This is the well known phenomenon of electrical endosmose which was first investigated experimentally by Quincke and Wiedemann and which was explained mathematically by Helmholtz. The earlier workers found that the watery phase of the double layer was generally positively electrified. Perrin<sup>5</sup> made the remarkable discovery that in the case of certain diaphragms, such as powdered charcoal, carborundum, gelatin, etc., the sign of charge can be reversed at will, chiefly with the aid of acid and of alkali. In a slightly acid medium the liquid moves to the anode, in a slightly alkaline medium it moves to the cathode. This has been confirmed by every observer, and his deductions have been generally accepted.

It has been suggested by Girard, Bernstein, Bartell and Hocker, and Freundlich<sup>6</sup> that the cases of so called negative osmosis where liquid diffuses from acid into pure water instead of in the opposite direction might be in reality manifestations of electrical endosmose. The only difference between the case of free osmosis and electrical endosmose being, according to these authors, the source of the potential difference, which is an external one in the case of electrical endosmose and an internal one—e.g. a diffusion or a boundary potential—in the case of free osmosis. But this is thus far merely an hypothesis which is not yet adequately supported by facts.

The possibility of correlating the phenomena of free and electrical osmosis meets at present with a difficulty. Our experiments on collodion membranes leave no doubt that in the case of *free osmosis* the influence of electrolytes on the velocity of diffusion of water from pure solvent to solution through the collodion membrane is an addi-

<sup>5</sup> Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50.

<sup>6</sup> Girard, P., *Compt. rend. Acad.*, 1908, cxvi, 927; 1909, cxviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401; La pression osmotique et le mécanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912. Bernstein, J., Elektrobiologie, Braunschweig, 1912. Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Freundlich, H., *Kolloid-Z.*, 1916, xviii, 11.

tive effect of the two oppositely charged ions. Perrin,<sup>6</sup> however, states that in the case of electrical endosmose only one of the oppositely charged ions of an electrolyte influences the transport of liquid through the membrane; namely, the one with the same sign of charge as that of the water (or with the opposite sign of charge from that of the membrane).

## II.

Perrin's<sup>5,7</sup> view of the influence of electrolytes on the amount of liquid transported in electrical endosmose rests on the assumption that the sign of the electrification of the double layer is primarily determined by the H and OH ions. He assumes that the positive electrification of a membrane bounded by liquid containing a monovalent acid is due to the adsorption of a layer of hydrogen ions by the membrane. This membrane layer of adsorbed hydrogen ions is the fixed stratum of the double layer and the next stratum of the liquid—the watery phase—contains a corresponding excess of negative ions. The stratum of negative ions is sufficiently far removed from the fixed layer so as to be able to undergo the tangential displacement on which the phenomena of electrical osmosis are supposed to depend.

Perrin assumes that the negative electrification of a membrane under the influence of a monovalent base is due to the OH ions situated in that stratum of the liquid which is in immediate contact with the membrane, while a corresponding excess of positive ions exists in the opposite stratum of liquid (the watery phase, in our terminology). When another electrolyte is added to a weak acid or weak alkaline solution the charge of the membrane is, according to Perrin, *influenced only by one of the two ions* of the electrolyte added; namely, the one with the opposite sign of charge from that of the membrane.

"When a liquid electrifies a membrane with a certain sign the addition to this liquid of a polyvalent ion of the same sign does not increase the electrification, while the addition of a polyvalent ion of the opposite sign diminishes this electrification considerably. The influence of bivalent ions is inferior to that of trivalent ions and that of trivalent ions is inferior to that of tetravalent ions. In the case of the ions with high valencies the diminution of the charge can result in the complete reversal of the sign of the charge."

<sup>7</sup> Perrin, J., Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918, 36-37.

His theory which has revolutionized colloid chemistry is expressed in the following statement.

"The primary factor of this electrification is always the action of the hydrogen or the hydroxyl ions, which are pressed against the membrane in the same way, no matter whether polyvalent ions are present or absent. But if polyvalent ions of the opposite sign are present they are attracted towards the membrane. To be more precise, let us suppose a liquid with a monovalent acid; hydrogen ions cover the membrane with a positive charge according to the degree of acidity. Behind them, at a distance which results from an equilibrium between the osmotic and the electric forces are found the monovalent negative ions forming the second stratum of the double layer. If we now add negative polyvalent ions, e.g.  $\text{Fe}(\text{CN})_6^-$ , the osmotic forces acting on the tetravalent  $\text{Fe}(\text{CN})_6^-$  remain of the same order as before while the electric force is multiplied by four; the density of the double layer will therefore diminish and as a consequence the P.D. of contact and the amount of electrical endosmose. It only remains to explain in a precise manner reversion of the sign of charge due to the presence of the necessary amount of  $\text{Fe}(\text{CN})_6^-$  ions."

Perrin's view on the effect of electrolytes on the double layer does not agree with our experiments with collodion membranes which show unequivocally that the influence of electrolytes on the rate of diffusion of water in the case of free osmosis is an additive effect of the two oppositely charged ions of an electrolyte, and not the effect of only one of the two ions.

If we assume that the influence of ions is the same in the case of free osmosis and in electrical endosmose an increase in the valency of the anion, according to Perrin, should not increase the rate of diffusion of positively electrified water in free osmosis, since in this case the membrane has the same sign of charge as the anion. A glance at Fig. 1 shows, however, that when we separate a solution from pure water by a collodion membrane the initial rate of diffusion of water into the solution increases in the lower concentrations of different potassium salts with increasing valency of the anion when the water is positively charged. In this case a watery solution of one of these salts was put into a collodion bag connected with a manometer and the bag was put into a beaker containing distilled water. The solutions of  $\text{NaCl}$ ,  $\text{CaCl}_2$ ,  $\text{Na}_2\text{SO}_4$ , and  $\text{Na}_4\text{Fe}(\text{CN})_6$  were rendered alkaline by dissolving the neutral salt in  $\text{M}/1,024$  or  $\text{M}/1,000 \text{ KOH}$ . The abscissæ are the logarithms of the concentration and the ordinates are the rise in the level of solution in the manometer after 20 minutes.

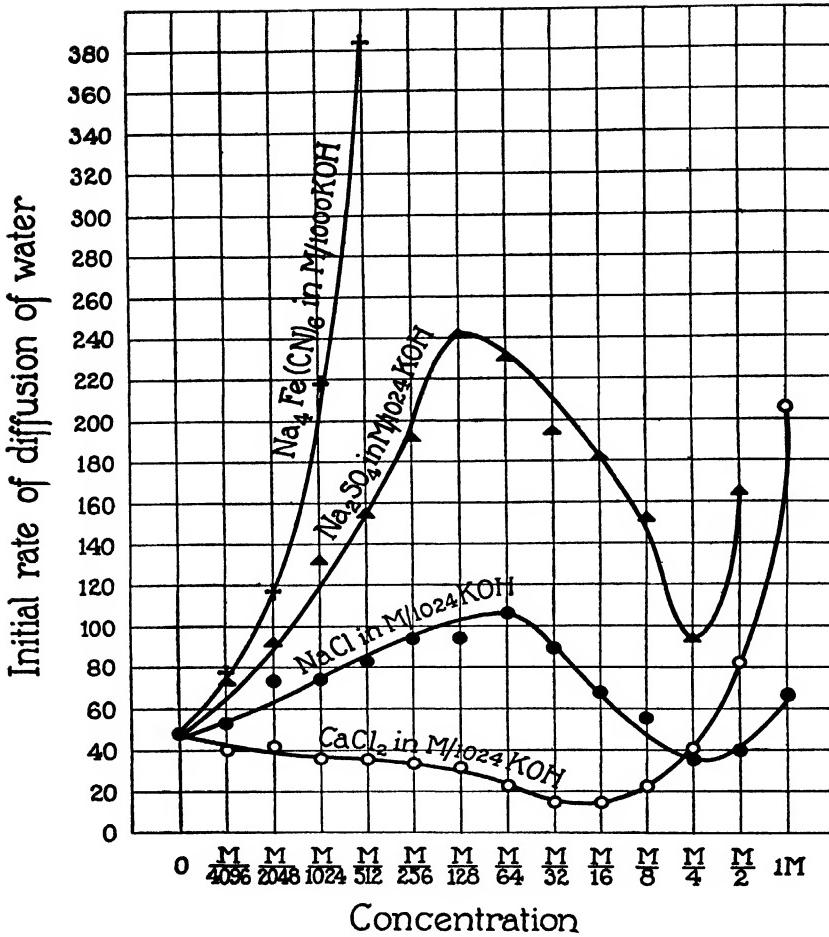


FIG. 1. Initial rate of diffusion of pure water through a collodion membrane into a solution containing an electrolyte. Abscissæ are the logarithms of concentration, ordinates rise of level of water in manometer connected with solution after 20 minutes. Solutions of salts were all rendered alkaline by enough KOH to make the solution about  $10^{-3}\text{N}$  in regard to KOH. The water was positively electrified. The curves show that the initial rate of diffusion of water into the solution increases with increasing valency of the anion of the electrolyte, though the membrane has the same sign of charge as the anion. The cations have a depressing effect, increasing also with the valency. The drop in the curve beyond a concentration of  $\text{M}/128$  or  $\text{M}/64$  is due to the fact that beyond this concentration the effect of the cation begins to prevail over that of the anion, until at a concentration of  $\text{M}/8$  or  $\text{M}/4$  the gas pressure effect of the electrolyte begins to prevail over the electrical effect.<sup>2,3,8</sup>

<sup>8</sup> Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 273.

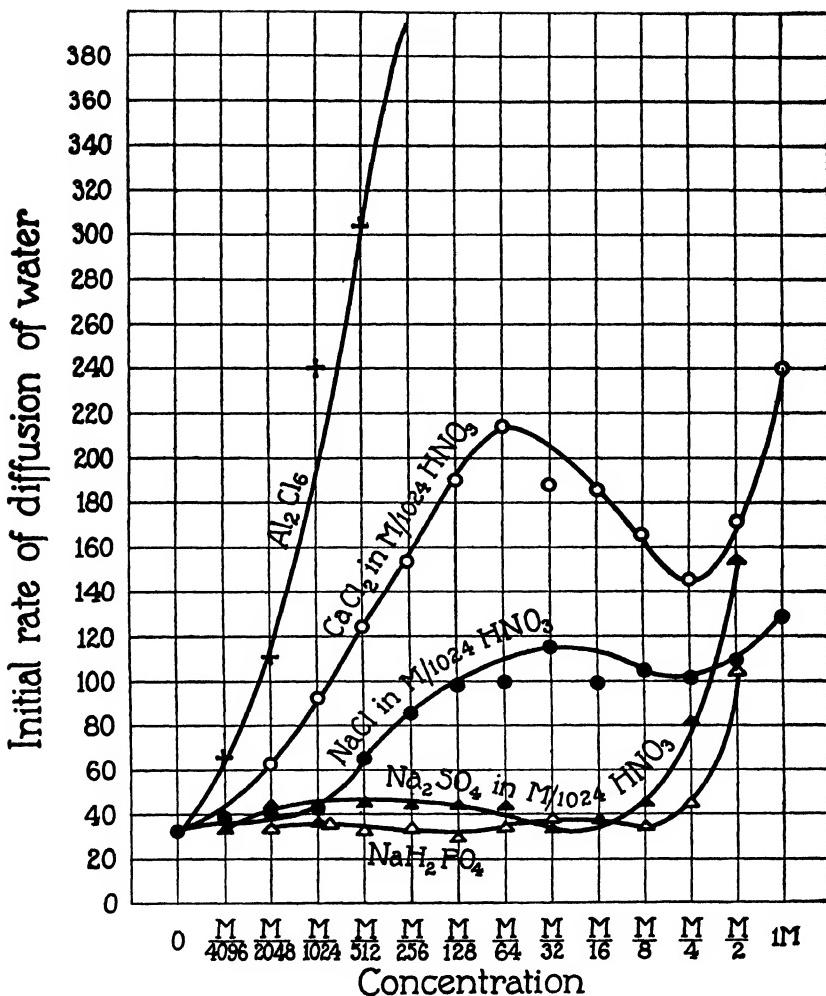


FIG. 2. Initial rate of diffusion of water from pure water through collodion membrane to solution of electrolytes rendered about  $10^{-3}$  N acid through addition of  $\text{HNO}_3$ . Though the water is negatively (and the membrane positively) electrified the initial rate of diffusion of water into the solution increases with increasing valency of the cation and diminishes with increasing valency of the anion. The drop in the curves in concentrations beyond  $M/64$  or  $M/32$  is due to the fact that in concentrations higher than these the depressing effect of the anion prevails over the opposite effect of the cation. For explanation of second rise of curve see legend of Fig. 1.

Moreover, according to Perrin, an increase in the valency of the cation should not increase the rate of diffusion of water from pure water into a slightly acid solution of salts, yet a glance at Fig. 2 shows that the initial rate of diffusion of water through a collodion membrane into a slightly acid solution increases considerably with the increase in the valency of the cation of the salt added.

In former papers,<sup>1, 2, 3</sup> further proof of these statements can be found and in addition the writer has shown that in neutral solutions (where the water diffusing through the collodion membrane is positively electrified) the rate of diffusion of water into the solution increases with the valency of the anion of the electrolyte in the solution.

This discrepancy between the actual observations concerning the influence of electrolytes on the rate of diffusion of water through a collodion membrane in free osmosis on the one hand, and the theory of Perrin concerning the influence of electrolytes on the rate of diffusion of liquid in the case of electrical endosmose on the other indicates that either the influence of electrolytes is not the same in both cases or that the theory of Perrin is not the correct expression of the facts in the case of electrical endosmose, at least for collodion membranes. It seemed, therefore, necessary to test the influence of electrolytes on the rate of transport of water through collodion membranes by electrical endosmose as a first step towards a theory of the influence of electrolytes on free osmosis.

### III.

In the experiments on electrical endosmose we used the collodion bags which served for the experiments on free osmosis. These collodion bags were cast inside an Erlenmeyer flask of a volume of about 50 cc. The collodion flask was closed with a rubber stopper which was perforated by a glass rod serving as a manometer. The bag was filled at the beginning of the experiment with the solution whose influence on the osmotic transport was to be investigated and was put into a beaker containing the identical solution. The bag was then so adjusted that the upper level of the rubber stopper was at the surface of the liquid in the beaker and that a column of liquid of about 30 mm. in the manometer was above the level of the liquid in the beaker. The manometer was a glass tube with a bore of about 2

mm. in diameter. One platinum electrode was put into this glass tube and one into the beaker. The distance between the electrodes was approximately the same in all experiments; namely, 6.7 cm. The electrode in the manometer was usually that pole towards which the transport of liquid occurred so that the rise of level in the manometer could serve as a measure for the volume of liquid transported. In this case the transport occurred against a hydrostatic pressure and it was necessary to compare the rise of level at about the same pressure head in different experiments.

The volume of liquid transported is a function of the external potential difference which was either 50 or 40 volts in our experiments. It turned out that in these experiments a disturbing variable entered inasmuch as with constant voltage the intensity of the current rose slowly and with the rise in intensity the amount of liquid flowing to one of the poles also rose gradually. The writer is inclined to interpret this gradual increase in intensity of current as being due to a gradual increase in the number of interstices through which the current can flow; and this means also an increase in the number of capillary spaces through which electrical endosmose can occur. Hence, for measurements of the transport of liquid only those changes in level could be used which occurred after the current had become approximately constant. In order to accelerate this process at the beginning of each experiment, a P.D. of 200 volts was used for 2 minutes or less until the intensity of the current was sufficiently high (above 1.0 or 2.0 milliamperes), and then the P.D. was lowered to the 40 or 50 volts desired. The voltage was then kept constant. As a measure of the effect of an electrolyte on the volume of liquid transported we used the rise in the level of liquid in the glass tube during the first 15 minutes after the current had become fairly constant. The following records will illustrate the way our figures were obtained. We omit the preliminary short treatment of the solution with a current of 200 volts, and give only the records for the 50 volt effects. The solutions used were  $\text{M}/512 \text{ KCl}$ ,  $\text{M}/512 \text{ K}_2\text{SO}_4$ ,  $\text{M}/512$  and  $\text{M}/1,024 \text{ K}_4\text{Fe}(\text{CN})_6$ ,  $\text{M}/512 \text{ CaCl}_2$ , and  $\text{M}/512 \text{ BaCl}_2$ . The solutions were almost neutral but slightly on the acid side of neutrality, the pH being about 6.2, and the transport of liquid occurred towards the cathode which was put into the glass tube. Table I gives the time in minutes, the in-

TABLE I.

*Transport of Liquid in Electrical Endosmose in Approximately Neutral Solutions,  
 $\text{pH} = 6.2$ . 50 Volts. Cathode in Capillary.*

Solution.	Time. min.	Millampere.	Rise of level of liquid in manometer.
			mm.
$\text{M}/512 \text{ KCl}$	0	1.3	+1.0
	5	1.7	+2.0
	10	1.9	+2.5
	15	2.1	+3.5
	20	2.15	+5.0
	25	2.18	+6.0
	30	2.18	+7.0
$\text{M}/512 \text{ K}_4\text{SO}_4$	0	1.0	+1.0
	5	1.4	+1.5
	10	1.9	+2.0
	15	2.4	+4.0
	20	2.3	+6.0
	25	2.3	+8.0
	30	2.25	+10.5
	35	2.25	+12.0
	40	2.26	+14.0
	45	2.2	+16.0
$\text{M}/1,024 \text{ K}_4\text{Fe}(\text{CN})_6$	0	1.0	+1.5
	5	1.45	+7.5
	10	2.0	+16.0
	15	2.35	+26.0
	20	2.45	+36.0
	25	2.45	+43.0
	30	2.6	+48.0
	35	2.7	+54.0
	40	2.76	+60.0
$\text{M}/512 \text{ K}_4\text{Fe}(\text{CN})_6$	0	1.4	+1.0
	5	2.4	+6.5
	10	3.4	+14.0
	15	3.85	+25.0
	20	4.0	+33.0
	25	4.2	+39.0
	30	4.2	+45.0
	35	4.2	+52.0
	40	4.4	+59.0

TABLE I—*Concluded.*

Solution.	Time.	Milliamperes.	Rise of level of liquid in manometer.
		min.	mm.
$M/512\ CaCl_2$	0	1.8	+3.0
	5	1.8	+3.0
	10	1.7	+3.0
	15	1.55	+2.0
	20	1.5	+1.5
	25	1.5	0
	30	1.5	-1.0
$M/512\ BaCl_2$	0	2.3	0
	5	1.6	-1.5
	10	1.2	-3.0
	15	1.15	-4.5
	20	1.15	-5.5
	25	1.1	-6.5
	30	1.15	-7.5

tensity of the current in milliamperes, and the rise in the level of the liquid in the glass tube.

In the case of  $M/512\ KCl$  the current became approximately constant after 15 minutes (2.1 to 2.2 milliamperes) and the rise in the level of liquid in the glass tube between 15 and 30 minutes, *i.e.* 7 — 3.5 mm. = 3.5 mm., was used as a measure for the relative influence of  $M/512\ KCl$  (approximately neutral) upon the rate of endosmotic transport of liquid.

The figures for transport during 15 minutes were selected as follows: for  $M/512\ K_2SO_4 = 10.5 - 4 = 6.5$ ; for  $M/512\ K_4Fe(CN)_6 = 45 - 25 = 20$ ; etc.

Since in the case of  $M/512\ K_4Fe(CN)_6$  the intensity of the current was greater than in the experiments with  $KCl$  or  $K_2SO_4$ , an experiment with  $M/1,024\ K_4Fe(CN)_6$  is added in the table. It gave 48 — 26 = 22 mm. as the transport number though the intensity of current was almost as low as in the case of  $M/512\ K_2SO_4$  or  $M/512\ KCl$ .

Experiments with neutral solutions of salts with bivalent cation, like  $CaCl_2$ ,  $BaCl_2$ , gave no transport with electrical endosmose even with 100 volts and an intensity of current of 4.8 milliamperes. Table II gives the transport numbers for various approximately neutral solutions.

Table II shows that the rate of endosmotic transport increases in almost neutral solutions of salts with increasing valency of the anion and diminishes with increasing valency of the cation. Electrolytes influence, therefore, the osmotic transport in exactly the same sense in free and in electrical osmosis. This influence is in both cases an additive effect of the oppositely charged ions of the electrolyte. In the presence of the salt mentioned the watery phase of the double layer is positively charged. The slight fall of level in the case of  $\text{CaCl}_2$  and of  $\text{BaCl}_2$  must be ascribed to the pressure head of about 30 mm. solution existing at the beginning of the experiment; since the same or a slightly more rapid fall of level occurs if no current passes through the liquid.

TABLE II.

*Relative Transport of Liquid by Electrical Endosmose in Approximately Neutral Solutions, pH = 6.2. 50 Volts.*

	Rise of level of liquid in manometer in 15 min.
	mm.
M/512 KCl.....	3.5
M/512 $\text{K}_2\text{SO}_4$ .....	6.5
M/512 $\text{K}_4\text{Fe}(\text{CN})_6$ .....	20.0
M/1,024 $\text{K}_4\text{Fe}(\text{CN})_6$ .....	22.0
M/512 $\text{CaCl}_2$ .....	0
M/512 $\text{BaCl}_2$ .....	0

We will now show that the statements made for neutral solutions are also true for alkaline solutions. In alkaline solutions the watery phase of the double layer is also positively charged and the liquid is transported to the cathode as in the case of neutral solutions. The cathode was put into the glass tube and the rise of level in the glass tube during the first 15 minutes after the current had become fairly constant was used as a measure for the transport. The solutions were brought to the same alkalinity as that of M/1,000 KOH and the pH varied between 10.9 and 11.0 (Table III).

The result is the same as before: the rate of endosmotic transport increases in alkaline solutions with increasing valency of the anion and diminishes with increasing valency of the cation. Electrolytes

influence, therefore, the transport of liquid in alkaline solution in the same sense in electrical endosmose as in free osmosis. This influence is in both cases an additive effect of the oppositely charged ions of the electrolyte. In the alkaline solutions the watery phase of the double layer is positively charged as it is in neutral solutions. If Perrin's rule applied to these experiments, the increasing valency of the anion should have had no effect.

Table III contains also the transport numbers of solutions of Na acetate, Na aluminate, and K oleate which are all higher than those of NaCl, although the anion is monovalent in each case. Solutions of

TABLE III.

*Transport of Liquid by Electrical Endosmose to the Cathode in Alkaline Solutions,  
pH = 10.9 to 11.0. 40 Volts.*

	Millampere.	Rise of level of liquid in manometer in 15 min.
		mm.
M/512 NaCl.....	1.1	3.5
M/512 Na <sub>2</sub> SO <sub>4</sub> .....	1.6	10.0
M/512 Na <sub>4</sub> Fe(CN) <sub>6</sub> .....	2.1	22.0
M/512 CaCl <sub>2</sub> .....	1.2	0
M/512 BaCl <sub>2</sub> .....	1.15	0
M/512 Na acetate.....	1.35	7.0
M/512 NaAlO <sub>2</sub> .....	1.15	7.0
M/512 K oleate (pH = 9.4).....	1.15	22.5

these salts also attract water more powerfully than solutions of NaCl in the case of free osmosis and the influence of these salts in electrical endosmose is parallel to their influence in free osmosis. These salts illustrate the statement that in addition to the valency another constitutional quantity of the ions determines their influence on the transport of liquid in free and electrical endosmose.

We finally investigated the electrical transport of liquid in acid solutions. The salt solutions were made N/1,000 acid by the addition of HNO<sub>3</sub>; the pH was in all cases exactly 3.0. Table IV gives the results. The anode was in the glass tube. In this case it was necessary to use membranes which had received a gelatin treatment.

If Perrin's rule applied to these cases, the increasing valency of the cation should not have influenced the result in these acid solutions.

We notice, however, that the rate of endosmotic transport to the anode increases in acid solutions with the increase in the valency of the cation and diminishes with the increase in the valency of the anion. Electrolytes influence, therefore, the transport of liquid in acid solutions in the same sense in the case of free and of electrical osmosis. The influence is in both cases an additive effect of the oppositely charged ions of the electrolyte. The watery phase of the electrical double layer is negatively charged in acid solutions of the hydrogen ion concentration used in this case; namely,  $10^{-3}$  N.

TABLE IV.

*Relative Transport of Liquid in Electrical Endosmose to the Anode in Acid Solutions,  
pH = 3.0. 40 Volts.*

	Millampere.	Rise of level of liquid in manometer in 15 min. mm.
M/512 NaCl .....	3.7	5.5
M/512 CaCl <sub>2</sub> .....	2.6	11.5
M/512 BaCl <sub>2</sub> .....	4.0	13.0
M/512 CeCl <sub>3</sub> .....	3.5	16.5
M/512 ThCl <sub>4</sub> .....	3.8	18.0
M/512 Na <sub>2</sub> SO <sub>4</sub> .....	3.4	0
M/512 Na <sub>2</sub> oxalate.....	3.2	0
M/512 NaH <sub>2</sub> PO <sub>4</sub> .....	1.8	2.0

If we summarize all three cases we may state that in both free and electrical osmosis, the transport of liquid is accelerated by that ion of an electrolyte which has the opposite sign of charge as the watery phase of the double layer (or the same sign of charge as the collodion membrane) and retarded by that ion which has the same sign of charge as the watery phase of the double layer (or the opposite sign of charge as the collodion membrane); and that both the accelerating and the retarding effect of ions increase with their valency and a second constitutional quantity of the ion which is still to be defined and for which the high transport number of several salts in Table III may serve as an example.

The writer has made a number of experiments on electrical endosmose with different concentrations of electrolytes. The curves repre-

senting this influence on the transport of liquid in electrical endosmose through collodion membranes seem to be similar to the curves, representing the influence of different concentrations of the same electrolytes on free osmosis, which were published in a preceding paper.<sup>3</sup>

In experiments with concentrations of electrolytes above M/512 or M/256 it is wiser to work with a lower voltage to avoid the excessive development of gas bubbles. Table V gives the numbers for the rise of liquid in the manometer for different concentrations of K<sub>4</sub>Fe(CN)<sub>6</sub>.

TABLE V.

*Influence of Concentration on Transport of Liquid by Electrical Endosmose to Cathode. 20 Volts.*

	Millampere.	Rise of level of liquid in manometer in 15 min.
		mm.
M/2,048 K <sub>4</sub> Fe(CN) <sub>6</sub> .....	0.1	1.5
M/1,024 K <sub>4</sub> Fe(CN) <sub>6</sub> .....	0.5	9.0
M/512 K <sub>4</sub> Fe(CN) <sub>6</sub> .....	0.9	10.5
M/256 K <sub>4</sub> Fe(CN) <sub>6</sub> .....	1.2	6.0
M/128 K <sub>4</sub> Fe(CN) <sub>6</sub> .....	1.8	3.0
M/64 K <sub>4</sub> Fe(CN) <sub>6</sub> .....	3.0	0

during 15 minutes after the intensity of the current had become fairly constant. The P.D. applied was 20 volts.

The maximum of transport of liquid in electrical endosmose was reached at a concentration of about M/512 K<sub>4</sub>Fe(CN)<sub>6</sub> and then the electro-endosmotic transport fell rapidly to zero with increasing concentration, although the intensity of the current increased with concentration. The drop in the curves representing the initial rate of diffusion of water from pure water to solution through collodion membranes in the case of free osmosis is therefore paralleled in the case of electrical endosmose (Table V). A fuller account of these results will shortly be published.

*Theoretical Remarks.*

According to the formula of Helmholtz for the transport of liquid by a current through capillaries, modified by Perrin,<sup>9</sup> we have

$$v = \frac{q \cdot \epsilon \cdot E \cdot D}{4 \pi \cdot \eta \cdot l} .$$

where  $v$  is the quantity of liquid carried electro-osmotically,  $\epsilon$  is the potential difference between the two strata of the double layer,  $E$  the external electromotive force,  $D$  the dielectric constant of the medium,  $\eta$  the coefficient of internal friction, and  $l$  the distance of the external electrodes. Since in our experiments all quantities occurring in this formula except  $v$  and  $\epsilon$  were kept approximately constant, we must attribute the influence of electrolytes on the quantity of transport  $v$  to an influence of the ions on  $\epsilon$ . We must therefore conclude that the influence of electrolytes on the rate of free osmosis is due to the effect of the ions of the electrolyte on the quantity of charge on the unit area of the Helmholtzian double layer. Our experiments on both free and electrical osmosis show that this influence is an additive effect of the two oppositely charged ions of the electrolyte at least in the case of collodion membranes. Since the quantity of transport  $v$  increases with the value of  $\epsilon$  we must further conclude that the ion with the same sign of charge as the watery phase of the double layer diminishes the value of  $\epsilon$  since this ion diminishes transport in both free and in electrical osmosis; while the ion with the opposite sign of charge as this watery phase increases the value of  $\epsilon$  in both forms of osmosis. Both effects increase with the valency and with the second constitutional quantity of the ion (Table III). The total effect of the two oppositely charged ions of an electrolyte on the rate of diffusion of water through a collodion membrane is therefore the difference between the opposite effects of its ions on the value of  $\epsilon$ . These statements give the theoretical basis of what we called in our former papers the apparent electrostatic action of the ions on the rate of diffusion of the electrified particles of water from pure water into solution through a collodion membrane (free osmosis).

<sup>9</sup> Freundlich, H., Kapillarchemie, Leipsic, 1909, 226.

Positively charged particles of water in the pores or interstices of the membrane will be driven to that side of the membrane which is more negatively charged. Since this is usually the solution side, water will be driven from the side of pure water into the solution.

When the collodion membrane has been treated with a protein, it is also generally negatively charged when bounded by water except when the solution contains hydrogen ions or simple trivalent or tetravalent cations beyond a certain concentration (which for H is  $10^{-4}$  N); in this case the membrane is positively and the watery phase is negatively charged. If we add in this case an electrolyte to the water, the charge on the membrane is increased by the cations and diminished by the anions of the electrolyte. Whenever the positive charge on the solution side of the membrane is greater than on the opposite side, the negatively charged particles of water will diffuse from the side of pure water to the side of solution.

When the charge on the solution side of the membrane is diminished by the electrolyte so that the charge is smaller than on the side of pure water, the liquid will flow through the membrane from solution side to the side of pure water (negative osmosis).

Our experiments were made with collodion membranes only and it is possible that Perrin's statement holds for other types of membranes. It seems, however, that in the case of the influence of electrolytes on the value of  $\epsilon$  at the boundary of oil drops and water the effect is also an additive one of the oppositely charged ions. Powis<sup>10</sup> has measured this value from the velocity of the motion of oil drops through solutions on the basis of the Helmholtz-Perrin formula for five electrolytes, KCl, BaCl<sub>2</sub>, AlCl<sub>3</sub>, ThCl<sub>4</sub>, and K<sub>4</sub>Fe(CN)<sub>6</sub>. The oil particles are negatively charged and their charge is increased more by K<sub>4</sub>Fe(CN)<sub>6</sub> than by KCl. The charge is diminished with the increasing valency of the cation. This indicates that the influence of electrolytes on the value of the potential difference of the double layer is in this case also an additive effect of the two kinds of ions.

We have stated that in the case of free osmosis the rate of diffusion of water from pure solvent to solution through a collodion membrane increases at first with increasing concentration, reaches a maximum

<sup>10</sup> Powis, F., *Z. physik. Chem.*, 1915, lxxxix, 91.

(which for many electrolytes lies at a concentration of about  $M/256$ ) and then drops again with a further increase in concentration. It seems from the writer's experiments that the same phenomenon occurs in the case of electrical endosmose through collodion membranes and that the turning point lies near  $M/512$ . Powis reports a similar effect of concentration in his observation on the motion of oil drops in an electrical field, and recently published experiments of Kruyt<sup>11</sup> on "current potentials" demonstrate the same phenomenon.

It seems to follow from this that the density of the electrical double layer at the boundary of watery phase and membrane increases at first with increasing concentration of an electrolyte up to a certain point which for a number of electrolytes seems to lie at about  $M/512$ . If the concentration of the electrolyte rises beyond this point, the density of the charge on the double layer diminishes rapidly with a further increase in the concentration of the electrolyte.

#### SUMMARY.

1. In three previous publications it had been shown that electrolytes influence the rate of diffusion of pure water through a collodion membrane into a solution in three different ways, which can be understood on the assumption of an electrification of the water or the watery phase at the boundary of the membrane; namely,

(a) While the watery phase in contact with collodion is generally positively electrified, it happens that, when the membrane has received a treatment with a protein, the presence of hydrogen ions and of simple cations with a valency of three or above (beyond a certain concentration) causes the watery phase of the double layer at the boundary of membrane and solution to be negatively charged.

(b) When pure water is separated from a solution by a collodion membrane, the initial rate of diffusion of water into a solution is accelerated by the ion with the opposite sign of charge and retarded by the ion with the same sign of charge as that of the water, both effects increasing with the valency of the ion and a second constitutional quantity of the ion which is still to be defined.

<sup>11</sup> Kruyt, H. R., *Kolloid-Z.*, 1918, xxii, 81.

(c) The relative influence of the oppositely charged ions, mentioned in (b), is not the same for all concentrations of electrolytes. For lower concentrations the influence of that ion usually prevails which has the opposite sign of charge from that of the watery phase of the double layer; while in higher concentrations the influence of that ion begins to prevail which has the same sign of charge as that of the watery phase of the double layer. For a number of solutions the turning point lies at a molecular concentration of about  $M/256$  or  $M/512$ . In concentrations of  $M/8$  or above the influence of the electrical charges of ions mentioned in (b) or (c) seems to become less noticeable or to disappear entirely.

2. It is shown in this paper that in electrical endosmose through a collodion membrane the influence of electrolytes on the rate of transport of liquids is the same as in free osmosis. Since the influence of electrolytes on the rate of transport in electrical endosmose must be ascribed to their influence on the quantity of electrical charge on the unit area of the membrane, we must conclude that the same explanation holds for the influence of electrolytes on the rate of transport of water into a solution through a collodion membrane in the case of free osmosis.

3. We may, therefore, conclude, that when pure water is separated from a solution of an electrolyte by a collodion membrane, the rate of diffusion of water into the solution by free osmosis is accelerated by the ion with the opposite sign of charge as that of the watery phase of the double layer, because this ion increases the quantity of charge on the unit area on the solution side of the membrane; and that the rate of diffusion of water is retarded by the ion with the same sign of charge as that of the watery phase for the reason that this ion diminishes the charge on the solution side of the membrane. When, therefore, the ions of an electrolyte raise the charge on the unit area of the membrane on the solution side above that on the side of pure water, a flow of the oppositely charged liquid must occur through the interstices of the membrane from the side of the water to the side of the solution (positive osmosis). When, however, the ions of an electrolyte lower the charge on the unit area of the solution side of the membrane below that on the pure water side of the membrane, liquid will diffuse from the solution into the pure water (negative osmosis).

4. We must, furthermore, conclude that in lower concentrations of many electrolytes the density of electrification of the double layer increases with an increase in concentration, while in higher concentrations of the same electrolytes it decreases with an increase in concentration. The turning point lies for a number of electrolytes at a molecular concentration of about  $M/512$  or  $M/256$ . This explains why in lower concentrations of electrolytes the rate of diffusion of water through a collodion membrane from pure water into solution rises at first rapidly with an increase in concentration while beyond a certain concentration (which in a number of electrolytes is  $M/512$  or  $M/256$ ) the rate of diffusion of water diminishes with a further increase in concentration.

## STUDIES OF ACIDOSIS.

### XV. CARBON DIOXIDE CONTENT AND CAPACITY IN ARTERIAL AND VENOUS BLOOD PLASMA.

By WILLIAM C. STADIE AND DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 29, 1919.)

According to the facts at our disposal, the bicarbonate content of the arterial blood plasma appears to be the blood figure most accurately indicating the alkaline reserve of the body fluids as a whole (Van Slyke and Cullen, 1917; Palmer and Van Slyke, 1917). In man it has heretofore been necessary to depend upon bicarbonate determinations in the venous blood as the closest practicable approximation to the arterial bicarbonate. In practice this has been estimated by determining the carbon dioxide capacity; that is, the CO<sub>2</sub> content of the plasma after saturation with air containing CO<sub>2</sub> at approximately the tension of normal alveolar air (Van Slyke and Cullen, 1917). The reliability of such determinations for the diagnosis of acidosis in metabolic diseases may be considered as demonstrated by the tests to which the method has been put in various laboratories, but it nevertheless remains desirable to compare the results thus obtained on the venous plasma with the actual arterial bicarbonate. The utilization of a technique for arterial punctures (Stadie, 1919) has rendered it possible to make this comparison in a series of patients, and the results are presented in this paper. Altogether thirty individuals were studied, most of whom had bronchopneumonia or lobar pneumonia of varying degrees of severity. A few normal individuals are included.

#### *Methods.*

The arterial blood was obtained as previously outlined (Stadie, 1919). The venous blood was taken without stasis, and, as a rule,

1 to 3 minutes after the arterial; both arterial and venous bloods were collected out of contact with air under albolene.

The CO<sub>2</sub> content and capacity were determined by the methods of Van Slyke (1917) and Van Slyke and Cullen (1917) respectively. The blood, collected under albolene to prevent loss or gain of CO<sub>2</sub>, was centrifuged, and 1 cc. samples of the plasma were withdrawn and discharged into the cup of the Van Slyke apparatus under a little ammonia to prevent escape of CO<sub>2</sub>. After thus determining the CO<sub>2</sub> content, the remaining plasma was saturated with air containing approximately 5.5 volumes per cent of CO<sub>2</sub>, and the CO<sub>2</sub> capacity then determined.

In calculating CO<sub>2</sub> capacity (by Table I, Van Slyke and Cullen, 1917) the CO<sub>2</sub> physically dissolved (H<sub>2</sub>CO<sub>3</sub>) is subtracted, so that the results represent only CO<sub>2</sub> bound as bicarbonate.

In calculating the CO<sub>2</sub> content, however (by Table I, Van Slyke, 1917), no subtraction for physically dissolved CO<sub>2</sub> is made, and the data represent total CO<sub>2</sub> from NaHCO<sub>3</sub> and H<sub>2</sub>CO<sub>3</sub> together. The free CO<sub>2</sub> in normal arterial plasma is about 3 volumes per cent; that is, a CO<sub>2</sub> content of 65 volumes per cent represents approximately 62 volumes per cent of bicarbonate CO<sub>2</sub> and 3 volumes per cent of free carbonic acid CO<sub>2</sub>.

The arterial oxygen unsaturation, or the percentage of hemoglobin in the arterial blood not combined with oxygen, was calculated as described by Lundsgaard (1918) using Van Slyke's method (1918) for the oxygen determinations. The oxygen content of the arterial blood was determined, and then a portion was thoroughly aerated, and the oxygen capacity determined.

$$\text{Per cent oxygen unsaturation} = \frac{\text{O}_2 \text{ capacity} - \text{O}_2 \text{ content}}{\text{O}_2 \text{ capacity}} \times 100.$$

In normal individuals at rest the arterial unsaturation averages about 5 per cent, 95 per cent of the hemoglobin in the arterial blood being saturated with oxygen. Figures for the unsaturation higher than 8 per cent indicate incomplete oxygenation of the arterial blood.

The results are given in Table I. In a few cases the arterial CO<sub>2</sub> content equals or slightly exceeds the venous. This may have been due to the short differences in time between the drawing of arterial and venous bloods, or to a summation of experimental errors in the

TABLE I.

Case No.	Arterial plasma.		Venous plasma.		A Arterial content Arterial capacity	B Arterial content Venous capacity	C Arterial content Venous content	Arterial oxygen unsatura- tion.  per cent
	CO <sub>2</sub> content.	CO <sub>2</sub> capacity.	CO <sub>2</sub> content.	CO <sub>2</sub> capacity.				
	vol. per cent	vol. per cent	vol. per cent	vol. per cent				
3	60.3	60.3	60.3	60.3				11.9
.	65.7	67.2	66.6	70.0	0.978	0.939	0.988	9.0
.	69.9		74.6	75.8		0.922	0.937	6.0
.	70.8	72.9	71.1	72.9	0.971	0.971	0.996	0
6	65.2		71.8	69.2		0.942	0.942	
8			67.3	73.7				8.1
9	50.3	56.8	50.8	54.9	0.886	0.916	0.990	68.2
10	69.0	73.0	68.4	71.6	0.945	0.964	1.009	4.6
11	57.3	62.4	59.2	64.9	0.918	0.883	0.968	10.1
.	62.6	67.5	66.0	68.9	0.928	0.909	0.948	8.9
12	59.5	66.5	63.8	69.4	0.895	0.857	0.933	6.3
.	56.2	61.7	60.1	64.6	0.911	0.870	0.935	13.7
13	63.5	66.2	62.5	66.2	0.959	0.959	1.016	2.8
.	61.9	62.1	64.7	65.0	0.997	0.952	0.957	7.5
14	63.2	70.0			0.903			20.7
15	55.5	62.3	60.9	66.3	0.891	0.837	0.911	7.8
.	63.1	65.3	67.4	69.1	0.966	0.913	0.936	7.3
16	55.8	59.4	59.7	66.7	0.939	0.837	0.935	14.1
17	59.6	62.2	60.5	62.2	0.958	0.958	0.985	16.3
.	69.1	71.6	77.6	78.1	0.965	0.885	0.891	11.5
18	56.0	63.3	57.8	63.3	0.885	0.885	0.969	16.6
.	61.4	64.0	59.0	64.0	0.959	0.960	1.041	16.5
.			63.1	70.8				38.2
19	63.9	68.4	65.8	68.4	0.934	0.934	0.971	7.9
.	57.1	76.5	61.8	76.0	0.746	0.751	0.924	0
20	55.7	62.0		61.1	0.898	0.912		13.4
22	63.3	70.8	71.1	75.5	0.894	0.838	0.891	19.5
.	46.8	51.9	51.5	50.0	0.902	0.936	0.909	25.1
23	66.8	62.1	69.7	69.6	1.076	0.960	0.959	15.1
.			67.2	78.1				8.9
24	64.5	66.2	71.2	68.1	0.974	0.947	0.906	2.4
25			54.9	50.9				44.1
26	63.7		71.9				0.886	5.2
30	50.2	52.6	57.3	65.8	0.954	0.763	0.876	24.9
33	55.5	60.0	58.4	57.2	0.925	0.970	0.950	23.4
34	58.8	61.4			0.958			14.9
.	53.3	57.2	53.3	59.2	0.932	0.900	1.000	33.5
.	56.7	58.8			0.964			25.9
.	56.1	57.8			0.971			10.7
38	56.2		60.6	63.2		0.889	0.927	8.4
39	57.4	61.0	59.3	66.8	0.941	0.859	0.968	14.5
40	59.8	59.5			1.005			
41	55.7	61.0			0.913			
42	50.8	51.4			0.988			
Average.....					0.938	0.904	0.950	

two determinations. The latter factor cannot be excluded because in many cases the amount of material did not permit analyses in duplicate. It is believed, however, that the number of determinations is sufficient to rule out the influence of such factors on the results viewed as a whole. The latter seem to justify the following conclusions.

#### CONCLUSIONS.

1. The carbon dioxide capacity ( $\text{NaHCO}_3$ ) of the venous blood plasma in man determined by the technique of Van Slyke and Cullen (1917) parallels the arterial plasma carbon dioxide content ( $\text{NaHCO}_3 + \text{H}_2\text{CO}_3$ ), which it exceeds on the average by about one-tenth. As the arterial  $\text{CO}_2$  is 95 per cent due to bicarbonate, the above results mean that the venous plasma carbon dioxide capacity parallels the arterial plasma bicarbonate, averaging about 115 per cent as great (Column B of Table I).
2. The carbon dioxide *content* of plasma from venous blood drawn without stasis parallels the arterial slightly more closely than does the venous carbon dioxide capacity and averages 105 per cent of the arterial content (Column C).
3. Consequently, for estimating the alkaline reserve in man, the venous plasma may be used for  $\text{CO}_2$  determination directly, without resaturation with carbon dioxide, if the blood is centrifuged and the plasma brought to analysis without opportunity for escape of  $\text{CO}_2$ .
4. Even when pulmonary conditions in pneumonia become so unfavorable for gas exchange that the arterial blood is incompletely oxygenated (high oxygen unsaturation), the arterial and venous carbon dioxide values are not increased above the usual normal levels. This might be expected from the fact shown by Krogh and Krogh (1910) that the lungs maintain approximate equality of carbon dioxide tension between arterial blood and alveolar air much more readily than they maintain equality of oxygen tension.

#### BIBLIOGRAPHY.

- Krogh, A., and Krogh, M., *Skand. Arch. Physiol.*, 1910, xxiii, 179.  
Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxii, 133.  
Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.

W. C. STADIE AND D. D. VAN SLYKE

Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215.

Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.



## A METHOD FOR THE DETERMINATION OF METHEMOGLOBIN IN BLOOD.

By WILLIAM C. STADIE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 29, 1919.)

In the course of experimental work on the production of methemoglobin in pneumococcic infections it became desirable to have some simple method for the determination of methemoglobin in blood. The author was unable to find in the literature any method other than the complex and time-consuming spectrophotometric method, which requires an elaborate apparatus and is not very suitable when many determinations have to be made. The method outlined below is simple, quickly performed, and has given satisfactory results in a study of methemoglobin formation which will be published shortly.

*Principle of the Method.*—The method depends upon the fact that both hemoglobin and methemoglobin are changed quantitatively to cyanhemoglobin by dilute solutions of potassium cyanide. The color of the latter is a brilliant orange-red and is very suitable for colorimetric comparison. The change of methemoglobin to cyanhemoglobin is rapid, even in the cold. Hemoglobin, however, changes slowly at room temperature, and must be heated to 50°C. for about  $\frac{1}{2}$  hour for complete conversion. That the cyanhemoglobin from hemoglobin is identical with that from methemoglobin is shown by the identical absorption spectra in the two cases.

In practice the difficulty arising from the slow conversion of hemoglobin into cyanhemoglobin is avoided by converting all the hemoglobin present into methemoglobin by the use of a little potassium ferricyanide, and then converting the methemoglobin into cyanhemoglobin. The resulting solution of cyanhemoglobin is compared with a standard of known strength in a Duboscq colorimeter.

The total amount of hemoglobin plus methemoglobin having been thus determined colorimetrically, the hemoglobin content of the blood containing the two pigments (hemoglobin and methemoglobin) is determined separately from the oxygen capacity, employing the technique outlined in Van Slyke's<sup>1</sup> gasometric determination of hemoglobin. The hemoglobin determined by the oxygen capacity is subtracted from the hemoglobin plus methemoglobin determined together as cyanhemoglobin; the difference is the methemoglobin.

*Details of the Method.*—Oxalated whole blood is used. 2.00 cc. of the blood are placed in a 100 cc. flask and 50 cc. of water are added which effect hemolysis in a few seconds. 0.5 cc. of a 0.1 M (3.0 per cent) solution of potassium ferricyanide is added, and the flask allowed to stand for 15 to 20 minutes. (It was found that these conditions are optimum for the complete conversion of the hemoglobin to methemoglobin, only the faintest visible hemoglobin

TABLE I.

*Factors for Calculating Results from Analysis of 2 Cc. of Blood Saturated with Air.*

Temperature. °C.	Air physically dissolved by 2 cc. of blood. Subtract from gas volume to obtain corrected gas volume representing O <sub>2</sub> set free from hemoglobin. cc.	Factor by which corrected gas volume is multiplied in order to give hemoglobin in 100 cc. of blood. gm.
15	0.037	$34.7 \times \frac{B}{760}$
16	0.036	34.6 "
17	0.036	34.3 "
18	0.035	34.2 "
19	0.035	34.0 "
20	0.034	33.9 "
21	0.033	33.7 "
22	0.033	33.5 "
23	0.032	33.4 "
24	0.032	33.1 "
25	0.031	33.0 "
26	0.030	32.9 "
27	0.030	32.6 "
28	0.029	32.5 "
29	0.029	32.3 "
30	0.028	32.1 "

<sup>1</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxii, 127.

band being present at the end of 20 minutes with this amount of potassium ferricyanide.) 5 cc. of a 0.1 per cent potassium cyanide solution are now added. The change to cyanhemoglobin is immediate. Water is added to the mark and the solution compared with a standard of known strength in a colorimeter. The result is the hemoglobin plus methemoglobin, which we express as gm. of "total hemoglobin" per 100 cc. of blood.

A small portion (4 to 5 cc.) of the blood or hemoglobin solution is aerated in a funnel and its total oxygen capacity determined by the Van Slyke method. Barcroft<sup>2</sup> has shown that under these conditions (180 mm. of oxygen tension, 0 mm. of carbon dioxide tension, and 100m temperature) the hemoglobin is practically 100 per cent saturated. Therefore the oxygen capacity corresponds to the amount of hemoglobin present, and by dividing by 1.34 (the volume of oxygen combined with 1 gm. of hemoglobin) we obtain the gm. of hemoglobin per 100 cc. of blood. For convenience of calculation the factors for the conversion of cc. of gas combined with 2 cc. of blood into gm. of hemoglobin per 100 cc. of blood are given in Table I (modified from Van Slyke<sup>1</sup>).

*Preparation of Standard.*—The standard is prepared from fresh whole oxalated or defibrinated blood which is known to contain no methemoglobin. The hemoglobin content (gm. per 100 cc.) is determined gasometrically. 500 cc. of standard are made by placing 10 cc. of blood in a 500 cc. flask, hemolyzing with about 300 cc. of water, and adding 2.5 cc. of the potassium ferricyanide solution. After 20 minutes, 25 cc. of the potassium cyanide solution are added and the mixture is diluted to the mark. The blood pigment value of this solution is known from the gasometric determinations and the unknown may be compared directly with it or suitable dilutions of the standard may be made.

*Calculation of Results.*—An example will make this clear.

Strength of standard 15.0 gm. of hemoglobin per 100 cc. of blood.

Comparison of cyanhemoglobin in colorimeter; Standard 10 Unknown 12.

Unknown has  $\frac{1}{2}$  of 15.0 or 12.5 gm. of total blood pigment per 100 cc.

Gasometric determination of hemoglobin 10.0 gm. per 100 cc.

Therefore, sample has 12.5–10.0 or 2.5 gm. of methemoglobin per 100 cc.

---

<sup>2</sup> Barcroft, J., *The respiratory function of the blood*, Cambridge, 1914.

## DISCUSSION.

The deep orange-red color of the cyanhemoglobin is adapted to accurate color comparisons. Fiftyfold dilution of normal blood, containing approximately 15 gm. of hemoglobin per 100 cc., gives about the optimum depth of color. A 1:100 dilution gives too light a color and requires that the standard be set at 20.

TABLE II.

Hemoglobin + methemoglobin observed per 100 cc. (Colorimetric.)	Hemoglobin calculated per 100 cc. (Gasometric.)	Methemoglobin observed per 100 cc.	Methemoglobin calculated per 100 cc.
gm.	gm.	gm.	gm.
6.89	6.74	0.15	0.00
6.89	6.43	0.31	0.46
6.86	6.15	0.69	0.71
6.62	4.56	2.17	2.06
6.58	0.20	6.54	6.38

The author has attempted to determine methemoglobin colorimetrically in blood by converting it, with the hemoglobin, into carbon monoxide hemoglobin, for estimation by the Palmer<sup>3</sup> method, and into acid hematin for estimation by the Sahli principle; but both attempts were unsuccessful, since methemoglobin does not form products with carbon monoxide or acid which can be colorimetrically compared with the products formed from hemoglobin.

*Results.*—A solution of hemoglobin obtained from sheep's cells previously washed with saline was used. A gasometric determination of this solution showed it to contain 6.74 gm. of hemoglobin per 100 cc. Part of this solution was shaken for  $\frac{1}{2}$  hour with an amount of potassium ferricyanide calculated to change all of it to methemoglobin. The hemoglobin content of this solution (gasometric) was 0.20 gm. per 100 cc. These two solutions were mixed in various proportions so as to make solutions containing varying but known amounts of hemoglobin and methemoglobin. The methemoglobin content was then determined as outlined above, using a

<sup>3</sup> Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

standard from ox blood which had been prepared a few days before. The results are shown in Table II.

#### CONCLUSIONS.

A colorimetric method for the determination of blood pigments is given which is simple and rapid, and which, combined with a simultaneous determination of the hemoglobin by the gasometric method of Van Slyke, gives the methemoglobin content of the blood.



[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, March 1, 1920, Vol. **xxi**, No. 3, pp. 267-273.]

## DETERIORATION OF CRYSTALLINE STROPHANTHIN IN AQUEOUS SOLUTION.

### ITS RELATION TO HYDROGEN ION CONCENTRATION AND A METHOD FOR ITS PREVENTION.

BY ROBERT L. LEVY, M.D., AND GLENN E. CULLEN, PH.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 7, 1920.)

For clinical use crystalline strophanthin is commonly dissolved in normal salt solution or water and marketed in glass ampules. Sterilization is accomplished by autoclaving after the ampules have been filled and sealed. In making biologic assays of several lots of a commercial preparation of ouabain (g-strophanthin) by the cat method of Hatcher and Brody<sup>1</sup> wide variations in potency were found. On adding a drop of indicator, phenol red, to the contents of the ampules showing low potency, it was observed that they were decidedly alkaline in reaction, whereas freshly prepared, aqueous solutions of the drug were neutral or slightly acid. Experiments were undertaken to ascertain the cause of the deterioration in relation to the altered hydrogen ion concentration and to devise a method for preparing a stable solution for therapeutic purposes.

#### *The Drug.*

Great variability in commercial preparations of crystalline strophanthin has been noted by Rowe,<sup>2</sup> who tested a number of lots bought on the open market by the "1 hour" frog method. The least active sample was 67 per cent as active as the standard (U. S. P.), whereas the most active was 240 per cent of the same standard, or about 3.5

<sup>1</sup> Hatcher, R. A., and Brody, J. G., The biological standardization of drugs, *Am. J. Pharm.*, 1910, lxxxii, 360. The cat unit represents the minimum lethal dose per kilo of cat, expressed in milligrams.

<sup>2</sup> Rowe, L. W., The variability of strophanthin, with particular reference to ouabain, *J. Am. Pharm. Assn.*, 1916, v, 1183.

times as potent. As indicated by Rowe, such variability in the crystalline product can, in large part, be attributed to the difficulty of obtaining unmixed seed of a desired variety, for there are more than twenty species of *Strophanthus* seed on the market and a given lot almost invariably contains specimens of several varieties. Further, different samples of the crystalline product contain variable amounts of water of crystallization; this, too, makes for differences in biologic activity expressed in terms of dry weight.

The drug employed in the present observations, as well as in a series of clinical studies to be reported later, was isolated from seeds purchased as *Strophanthus hispidus*, but apparently actually *Strophanthus gratus*. The procedure of isolation followed was based on the methods of Heffter and Sachs, and Thoms.<sup>3</sup>

The ground seeds were freed from fat with petroleum ether and the dried residue was extracted with cold 70 per cent alcohol. The extract was concentrated to a thick mass *in vacuo*, taken up in hot water, treated with hot basic lead acetate until no further precipitate formed, and filtered. The excess of lead was precipitated from the filtrate by means of a little ammonium sulfate and filtered off. The filtrate was saturated with ammonium sulfate, which precipitated a mixture of the amorphous and the crystalline strophanthin (ouabain), the last portions of the latter separating in crystalline form. The precipitate was dissolved in warm water, filtered, and the filtrate chilled and seeded with a few strophanthin crystals. After being allowed to stand in the ice box, the crystalline strophanthin was filtered off and recrystallized from water.

The crystals so obtained consisted of transparent, glistening platelets, containing 8.5 molecules of water of crystallization. The color reactions corresponded to those found by Arnaud and by Thoms.<sup>4</sup>

Physical constants:—Anhydrous substance:  $[\alpha]_D^{20} = -30.2^\circ$

Calculated for  $C_{30}H_{46}O_{12}$ : C, 60.17; H, 7.75

Found: C, 60.05; H, 7.81

Melting point (uncorrected): Softens: above  $185^\circ\text{C}$ ;  
melts to paste:  $187\text{--}188^\circ\text{C}$ .; evolves gas:  $195\text{--}197^\circ\text{C}$ .

<sup>3</sup> For the isolation and identification of the drug we are indebted to Dr. W. A. Jacobs and Dr. M. Heidelberger, of The Rockefeller Institute. They have also kindly furnished us with notes of the methods employed by them in these procedures. See also Heffter, A., and Sachs, F., *Vergleichende Untersuchungen über Strophanthus-Glucoside*, *Biochem. Z.*, 1912, xl, 83; Thoms, H., *Die Strophanthus-Frage vom chemischen Standpunkt*, *Ber. pharm. Ges.*, 1904, xiv, 104.

<sup>4</sup> Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 104.

Biologic standardizations of this crystalline strophanthin were carried out on thirty-one cats, and in twenty-six of the animals electrocardiograms were made at frequent intervals during the course of the experiments in order to determine the physiological effects of the glucoside upon the cat's heart. The average cat unit was found to be 0.104 mg. per kilo, a figure in accord with the standard of 0.10 mg. per kilo established by Hatcher and Brody. The electrocardiographic findings confirmed in their essentials the observations of Levine.<sup>5</sup> Chemically, biologically, and physiologically, therefore, the strophanthin at our disposal possessed those properties ascribed to the crystalline product obtained from *Strophanthus gratus* and usually marketed under the name "ouabain."

#### *Experiments with Glassware.*

Doubly distilled water, pH 6.0, was autoclaved for 20 minutes at 15 pounds pressure in various types of glass bottles and flasks, chosen at random from the laboratory supply. Immediately after autoclaving, the reaction of the water in the cheaper and softer varieties of container had become distinctly more alkaline, the pH ranging from 6.3 to 9.0+.<sup>6</sup> In the hard glass flasks (Pyrex) no significant alteration in reaction (less than 0.1) occurred. A standard 0.02 M phosphate mixture, pH 7.0,<sup>7</sup> autoclaved in the bottles giving off the maximum amount of alkali in the process of sterilization, showed no alteration in hydrogen ion concentration, the buffer action being adequate to compensate for the dissolved alkali.

A similar experiment was done with sixteen types of glass ampules obtained from a number of pharmaceutical firms and used by them in marketing their products. The ampules were filled with distilled water, sealed, and autoclaved. In every instance the water in the

<sup>5</sup> Levine, S. A., The action of strophanthin on the living cat's heart, *J. Exp. Med.*, 1919, xxix, 485.

<sup>6</sup> Determinations of hydrogen ion concentration were done colorimetrically. In the more alkaline solutions the results obtained by the colorimetric method were checked by the use of the hydrogen electrode.

<sup>7</sup> The standard phosphate mixtures used were prepared according to the directions of Sörensen (Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 201; *Ergebn. Physiol.*, 1912, xii, 393).

ampules showed a change in pH, which now ranged from 6.2 to 9.0+. In order to titrate back to neutrality (pH 7.0) the most alkaline solution in the series, with phenol red as indicator, 2.6 cc. of 0.02 N hydrochloric acid per 100 cc. were required (Table I).

TABLE I.

*Hydrogen Ion Concentration of Distilled Water after Autoclaving in Various Types of Glass Ampules. Initial pH = 6.0.*

Ampule No.	pH of contained water after autoclaving 20 min.	Amount of 0.02 N HCl required to titrate 100 cc. of water to pH 7.0.* cc.
1	6.7	
2	6.2	
3	7.0	
4	7.0	
5	7.0	
6	7.0	
7	7.0	
8	9.0+	1.10
9	7.0	
10	7.2	
11	7.0	
12	9.0+	2.17
13	7.0	
14	9.0+	1.35
15	9.0+	2.10
16	9.0+	2.60

\* Phenol red was used as indicator.

*Effect of Changes in Hydrogen Ion Concentration on Aqueous Solutions of Crystalline Strophanthin.*

A 2 per cent solution of strophanthin was made in standard 0.05 M phosphate mixtures with pH 5.0, 7.0, and 8.6. Biologically tested, the cat unit of these solutions was found to be 0.107 mg. per kilo,<sup>8</sup> their optical rotation - 0.97°. After autoclaving for 20 minutes at

<sup>8</sup> In making determinations of cat unit values at least three animals were employed to calculate the biologic activity. Each figure given, therefore, represents the average of three or more cat experiments. Where it is stated that no alteration in activity was noted, it is meant that less than a 10 per cent variation occurred, this variation representing changes in the third decimal place only.

15 pounds pressure in Pyrex flasks, no alteration in optical activity and no significant change in potency were observed in the acid or neutral solutions. The alkaline mixture, however, now had an optical rotation of  $-0.93^\circ$  and a cat unit value of 0.152. In short, when strophanthin was autoclaved in alkaline solution (pH 8.6), the molecule was partially decomposed, with resultant alteration in its ability to rotate polarized light and significant reduction in biologic activity (Table II).

The degree of alkalinity attained by aqueous solutions autoclaved in soft glass ampules is, therefore, more than ample to cause chemical changes in their contents.

TABLE II.

*Efect on Biologic Activity and Optical Rotation of Autoclaving Crystalline Strophanthin in 2 Per Cent Aqueous Solution at Different Hydrogen Ion Concentrations.*

pH of 0.05 M phosphate solution.	Cat unit value per kilo.		Optical rotation.	
	Before autoclaving.		After autoclaving.	
	mg.	mg.	Before autoclaving.	After autoclaving.
5.0	0.107	0.104	$-0.97^\circ$	$-0.97^\circ$
7.0	0.107	0.105	$-0.97^\circ$	$-0.97^\circ$
8.6	0.107	0.152	$-0.97^\circ$	$-0.93^\circ$

For clinical use it is convenient to employ crystalline strophanthin in dilute concentration, usually 0.01 per cent. Such solutions autoclaved in soft glass ampules, which gave off enough alkali to alter the reaction of the contents from pH 6.0 to pH 9.0, became at once biologically inert, more than four times the calculated lethal dose having no appreciable effect on the cat's heart. The contents of hard glass ampules, with no significant alteration in pH after sterilization (0.1 or less), retained full potency. In hard glass (Pyrex) such autoclaved solutions have been kept without deterioration for over 5 months.

Holste<sup>9</sup> noted an alkaline reaction in solutions of Boehringer's amorphous k-strophanthin which had deteriorated, and suggested that alkali given off from the glass containers might have induced hydrolytic cleavage of the glucoside.

<sup>9</sup> Holste, A., Zur Strophanthinfrage, *Z. exp. Path. u. Therap.*, 1918, **xix**, 153.

Like Thoms,<sup>10</sup> who observed marked reduction in toxicity for mice of sterile aqueous solutions of amorphous g-strophanthin, he found crystalline g-strophanthin to be more stable. Holste's observations, however, were carried no further, and no method for preventing deterioration was offered.

We have had made for our use tablets of g-strophanthin, 0.5 mg., triturated with sugar of milk. The amount of drug contained is so small and the percentage error in measuring out the powder for the individual tablet is, therefore, relatively so large, that this form of preparation was discarded as unsatisfactory.

*A Method for Preparing Crystalline Strophanthin in Solution for Clinical Use.*

It is clear that stability of reaction is essential if the potency of strophanthin in solution is to be preserved. The glass commonly used in the manufacture of ampules for commercial purposes is, in large part, of the soft variety, yielding considerable amounts of alkali to solutions contained in it. It is advisable, therefore, to put up solutions of crystalline strophanthin in hard glass containers. Ampules of Pyrex glass have proved satisfactory. In order to avoid even slight changes toward the alkaline side, the drug may best be prepared in 0.02 M standard phosphate solution at pH 7.0.<sup>11</sup> This concentration of phosphate is harmless when injected intravenously in small amounts (5 to 10 cc.) into patients and does not interfere with either the chemical or physiological properties of the glucoside. Crystalline strophanthin in such a solution, in 0.01 per cent concentration (5 cc. contain 0.5 mg.), sealed in ampules, and autoclaved, has been preserved without evidence of reduction in biologic activity for more than 5 months.

<sup>10</sup> Thoms, H., Alte und neue Aufgaben der pharmazeutischen Chemie und insbesondere über die biologische Prüfung der Arzneimittel, *Ber. pharm. Ges.*, 1913, xxiii, 452.

<sup>11</sup> A 0.02 M phosphate solution at pH 7.0 contains 1.03 gm. of  $\text{KH}_2\text{PO}_4$  and 1.76 gm. of  $\text{Na}_2\text{HPO}_4$  (anhydrous) per liter.

## SUMMARY.

1. Many of the glass containers commonly used in the laboratory, and most of the glass ampules employed in marketing sterile solutions for hypodermic or intravenous medication, yield sufficient alkali on autoclaving, to change the reaction of distilled water from pH 6.0 to pH 9.0.
2. This increase in alkalinity is sufficient to render biologically inert and partially to decompose aqueous solutions of crystalline strophanthin in the concentration ordinarily employed in the clinic.
3. It is suggested that for clinical use crystalline strophanthin be dissolved in 0.02 M standard phosphate solution at pH 7.0, and marketed in hard glass ampules, thereby insuring stability of reaction with preservation of biologic activity.
4. It should be borne in mind, both by laboratory worker and pharmacist, that the alkali yielded, on heating, by soft glass containers may be responsible for a considerable alteration in the hydrogen ion concentration of their contents.



[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, March, 1920, Vol. xli, No. 3,  
pp. 345-348.]

## DETERMINATION OF CHLORIDES IN WHOLE BLOOD.

By J. HAROLD AUSTIN AND DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 26, 1920.)

As it is desirable at times to determine the chloride content of the whole blood rather than of the plasma only, the applicability to this purpose of the Van Slyke-Donleavy method<sup>1</sup> for the determination of plasma chlorides was investigated.

It was found that some component of the laked cells other than the chloride has the property of binding silver, so that the direct application of the Van Slyke-Donleavy method to whole blood gives readings too high by 30 to 40 per cent. If, however, after laking, the protein is precipitated by picric acid alone, or by picric and nitric acids, and the protein-free filtrate is treated with silver nitrate, a quantitative precipitation of the total chloride of the whole blood is obtained and the final titration may be carried out as in the Van Slyke-Donleavy method. The accuracy of this modification was tested on whole oxalated ox blood by comparison with chloride estimation by the Carius method, using the technique for destroying the organic matter adopted by Vinograd<sup>2</sup> and a Volhard titration of the silver remaining unbound.

### *Carius Method.*

Whole oxalated ox blood was analyzed for chloride content by the Carius method as follows.

Approximately 1 cc. of the whole blood was introduced from a pipette into the bottom of a bomb tube and the amount accurately determined by weighing the pipette before and after delivery. 1 cc.

<sup>1</sup> Van Slyke, D. D., and Donleavy, J. J., *J. Biol. Chem.*, 1919, xxxvii, 551.

<sup>2</sup> Vinograd, M., *J. Am. Chem. Soc.*, 1914, xxxvi, 1548; also *Studies from The Rockefeller Institute for Medical Research*, 1915, xxii, 372.

of a solution of silver nitrate containing 20.4 mg. of  $\text{AgNO}_3$  per cc. was carefully introduced into the bottom of the tube. The tube was then immersed in a water bath at 100° and the moisture-laden air in the upper portion of the tube continually removed by aspiration until the water was driven from the mixture of blood and silver nitrate. 1 cc. of fuming nitric acid in a small tube was then introduced into the tube. The tube after being sealed was slowly heated to 180° and this temperature maintained for 3 hours. After cooling, opening, and washing out the colorless solution with distilled water the remaining unprecipitated silver was titrated by Volhard's method with a 0.015 M solution of  $\text{NH}_4\text{CNS}$ , the chloride obtained by difference being expressed as  $\text{NaCl}$ . The results of four determinations are given in Table I.

*Results with Modified Van Slyke-Donleavy Method.*

Into each of two 50 cc. volumetric flasks were introduced 4 cc. of the same whole oxalated ox blood used in the Carius analysis and the weight of blood introduced was determined by weighing the flasks before and after introduction of the blood. After laking by addition of 20 cc. of distilled water, there were added 20 cc. of the following solution of nitric and picric acids:

	cc.
$\text{HNO}_3$ (sp. gr. 1.42) .....	250.0
Picric acid .....	7.5
Distilled water to .....	1,000.0

The flask was then filled to the mark with distilled water and repeatedly inverted. After allowing 10 minutes for complete precipitation of the protein, the mixture was filtered through a dry filter and 25 cc. of the clear yellow filtrate were introduced into a 50 cc. volumetric flask. To this were added 10 cc. of an M/29.25  $\text{AgNO}_3$  solution, the flask was filled to the mark, and two drops of caprylic alcohol were added. After standing over night (to promote clear filtration) the clear supernatant fluid was removed with a pipette, filtered, and two 20 cc. portions were taken. These were titrated with KI solution after addition of 4 cc. of the special starch solution as described in the Van Slyke-Donleavy method.

The results of four determinations are given, together with those on the same blood by the Carius method, in Table I. In the last column are given also the results obtained in attempting to apply the Van Slyke-Donleavy method, without modification, to whole blood.

The chief difficulty encountered in the present method was in securing a clear filtrate after the precipitation of the AgCl. This is proba-

TABLE I.

*Results of Chloride Determinations on a Specimen of Whole Blood.*

Carius method.			Van Slyke-Donleavy method modified for whole blood by precipitating proteins and AgCl separately.		Van Slyke-Donleavy method applied to plasma applied to whole blood without modification.
Weight of blood. gm.	NaCl found. mg.	NaCl found per gm. of blood. mg.	Weight of blood. gm.	NaCl found per gm. of blood. mg.	NaCl found per gm. of blood. mg.
0.998	4.47	4.48	4.108	{ 4.53 4.53	6.00
1.000	4.54	4.54			5.95
1.005	4.57	4.55	4.113	{ 4.54 4.53	5.98
1.002	4.57	4.56			6.05
Average.....		4.53		4.53	5.99

bly somewhat facilitated by using more picric acid and less nitric acid for the precipitation of the protein, then adding the required amount of nitric acid to the final 20 cc. portions just before addition of the starch solution. The most effective means of securing a clear filtrate, however, is by permitting the solution to stand in the dark over night after the addition of the silver nitrate; a perfectly clear supernatant liquid can then be pipetted from the flask.

The technique finally adopted is as follows.

*Method for Determination of Chlorides in Whole Blood.*

Take 3 cc. of blood with 15 cc. of water in a 60 cc. flask. Add 30 cc. of saturated picric acid solution and sufficient water to bring the volume to 60 cc. (27 cc. of water in all, and 30 cc. of picric acid may be measured from burettes if a 60 cc. measuring flask is not available). The contents of the flask are mixed, and after 10 minutes are filtered.

To 40 cc. of the filtrate 10 cc. of the M/29.25 silver nitrate solution used by McLean and Van Slyke<sup>3</sup> are added, with two drops of caprylic alcohol (the silver solution contains per liter 5.812 gm. of AgNO<sub>3</sub> and 250 cc. of HNO<sub>3</sub> (sp. gr. 1.42)). The solutions are thoroughly mixed, and preferably allowed to stand over night to allow the AgCl to coagulate and settle. The supernatant solution is decanted through a small filter paper, and 20 cc. are titrated as described by Van Slyke and Donleavy. The calculation also is the same, since the 20 cc. of filtrate titrated in this case, as in the method of Van Slyke and Donleavy, represent 0.8 cc. of the original material, blood or blood plasma.

Attention may be called to the fact that the use of the unmodified Van Slyke-Donleavy method with a plasma that is considerably stained as the result of partial hemolysis of the cells will give values that are unduly high.

## CONCLUSIONS.

1. The method described by Van Slyke and Donleavy for the determination of plasma chlorides is not directly applicable to whole or to laked blood.
2. A modified technique for whole or laked blood is described in which the precipitation and removal of the protein precede the addition of the silver.

<sup>3</sup> McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, **xxi**, 361.

[Reprinted from the AMERICAN JOURNAL OF DISEASES OF CHILDREN, February, 1920,  
Vol. xix, No. 2, pp. 97-113.]

## CALCIUM METABOLISM OF INFANTS AND YOUNG CHILDREN, AND THE RELATION OF CALCIUM TO FAT EXCRETION IN THE STOOLS.

### PART I. INFANTS TAKING MODIFICATIONS OF COW'S MILK.

By L. EMMETT HOLT, M.D., ANGELIA M. COURTNEY, AND HELEN L. FALES.  
*(From the Laboratories of The Rockefeller Institute for Medical Research and the Babies' Hospital.)*

Recently a large number of stools from children who were receiving food of a varied character were collected for a study of fat metabolism of infants and young children. In nearly all of this material total ash and calcium determinations were made. It was believed that the large number of observations made would furnish data of value in answering some much debated questions regarding calcium metabolism, especially in its relation to fat metabolism.

Many investigators have published data and have advanced theories on this subject, but the conclusions reached are contradictory and in many cases the observations on which they are based are very few in number. For example, the observations made on one breast fed infant for a single period and on one child fed on cow's milk, also for a single period, are quoted in nearly every article on this subject, and have been taken as standards for normal calcium metabolism.

Many diverse views are prevalent in regard to calcium metabolism. Some writers believe that a liberal intake of calcium results in an excessive excretion of calcium which is accompanied by a harmful loss of fat. Others hold that a high fat intake induces a large fat excretion, accompanied by a serious loss of bases, especially calcium. Both these dangers are supposed to be associated with occurrence of soapy stools. The German literature especially has emphasized the pathologic significance of large light-colored crumbly stools rich in calcium soaps and calcium phosphate, a type of stool apparently not so frequently seen in this country. There are still other writers who think that a large intake of calcium leads to a storage of calcium in

the body which may have injurious effects. On the other hand, there are many who believe that an ample intake of both fat and calcium produces only beneficial results. It is generally accepted that cod liver oil has a beneficial effect on the retention of calcium, but this opinion seems to be not entirely unanimous. The literature on all these questions is so voluminous that there is not space here to discuss it in detail.

The points of general interest in regard to calcium metabolism may be stated in the form of the following questions.

1. What is the normal excretion and absorption of calcium by children taking (a) modifications of cow's milk, (b) mixed diet?
2. How is the calcium excretion and absorption affected by the amount of (a) the calcium intake, (b) the fat intake?
3. Is there any evidence that either an excessive calcium intake or a very small calcium intake is harmful?
4. Is there any relation between the age or the weight of the child and the amount of calcium absorbed normally?
5. Is there any constant relation between the excretion of calcium and the excretion of fat in the form of soap?
6. Is there a serious loss of calcium in soapy stools?
7. On what is the calcium percentage of the total solids of the stool dependent?
8. How do the calcium excretion and absorption differ from normal when children are suffering from (a) diarrhea, (b) chronic intestinal indigestion, (c) active rickets or (d) when recovering from rickets?
9. What is the effect on calcium metabolism of (a) cod liver oil, (b) of vegetable fats?

In the observations reported in this and a subsequent paper on this subject the period of stool collection and the amount of food intake were exactly known. Thus, it was possible to determine closely the amount of calcium excreted and absorbed daily. In most of these cases values for the urinary excretion of calcium were not obtained. Hence, the actual *retention* of calcium is not reported. However, the *absorption* is practically the same as the retention, since the excretion of calcium in the urine of infants and young children is normally very small in amount. Previous work done in this laboratory has shown a general range of from 0.01 to 0.05 gm. of calcium oxid ( $\text{CaO}$ ) excreted

daily in the urine of infants taking modifications of cow's milk and from 0.03 to 0.15 gm. for children taking a mixed diet. With the small number of breast fed infants for whom values have been obtained, the urinary excretion of calcium was found to be from 0.014 to 0.026 gm. of calcium oxid daily. Rachitic children were found to excrete even less calcium oxid in the urine than did normal children of the same age. By calcium *excretion* is meant the total amount of calcium lost in the stools. Whether part of this calcium has been absorbed and subsequently excreted into the large intestine, as is believed by many to be the case, is not taken into consideration.

By calcium *absorption* is here meant the difference between the calcium intake and the excretion of calcium in the stools.

The values for ash and calcium in the feces were obtained by analysis of the dried material. The ash was determined by the Stölte method,<sup>1</sup> which consists of the application of heat to the finely ground material in a platinum dish inside a porcelain or silica dish, without the addition of acid or alkali. The calcium was determined in the weighed ash by the McCrudden method.<sup>2</sup>

In some of the cases, especially when the children were taking a mixed diet, the calcium in the food was not determined by direct analysis, but was estimated from the known weight or measurement of the various articles of the food intake. The calcium content of some of the articles of food which made up the diet had previously been determined in this laboratory.

In this article are presented only the findings for infants taking modifications of cow's milk. The data for children taking mixed diet will be discussed in a later paper.

### *Healthy Infants.*

The first group of infants here considered were normal or approximately normal as to their digestion. Most of these children were healthy and gaining weight at the time of observation. None of them had diarrheal stools. Table 1 shows the relation of the calcium absorption to the calcium and the fat intake for the group. These

1. Stölte: Biochem. Ztschr. 35: 104, 1911.

2. McCrudden: J. Biol. Chem. 10: 187, 1911.

TABLE 1.  
Normal Infants. Relation of Absorption of Calcium to Calcium and Fat Intake.

No.	Case	Age in Mo.	Weight in Gm.	Stools	Intake of CaO, Gm. per Kg.	CaO Absorbed, Gm. per Kg.	Per Cent. of CaO Absorbed	Gm. Intake of CaO per Gm. Fat Intake	Remarks
113	J. S. 1	7.5	4,060	Nonhomogeneous Constipated	0.498	0.099	20.2	6.6	0.074 Skinned milk with large amount of olive oil
72	A. K.	5.5	3,910	Normal	0.297	0.133	44.8	5.6	0.053
58	J. I.	12	5,485	Constipated	0.285	0.131	46.1	5.2	0.055
65	P. D. 3	7.5	4,884	Normal	0.285	0.068	23.8	4.9	0.058 Rather high carbohydrate in diet
54	R. C.	8.5	6,290	Normal	0.283	0.124	43.8	5.2	0.054 High calories per kilogram
86	J. M.	4	3,662	Soft, normal	0.279	0.098	35.3	3.7	0.076 Dried milk formula
158	H. B.	13	6,935	Normal	0.266	0.043	16.4	5.0	0.053 Very large stools
57	M. S. 2	8	6,145	Normal	0.266	0.123	46.6	4.8	0.055
70	P. D. 1	7	4,635	Constipated	0.264	0.095	38.5	4.9	0.053 Rather high carbohydrate in diet. High calories per kilogram
71	P. D. 2	7	4,679	Constipated	0.257	0.030	11.7	4.8	0.053 Rather high carbohydrate in diet. High calories per kilogram
82	P. L.	4	5,055	Nonhomogeneous Soft, normal	0.235	0.099	42.0	3.1	0.076 Dried milk formula
97	B. W. 1	15	5,873	Normal	0.231	0.117	50.8	4.7	0.049 Recovering from multiple fractures. Very low calories per kilogram
85	C. M.	2	3,450	Nonhomogeneous	0.229	0.116	50.7	4.2	0.055
166	A. A.	7	3,489	Nonhomogeneous	0.224	0.063	28.2	3.3	0.068 Very high proportion carbohydrate in diet
63	F. N. 2	11	5,800	Soft, normal	0.222	0.093	41.9	4.2	0.054 High carbohydrate in diet. High calories per kilogram
60	F. N. 1	11	5,620	Constipated	0.217	0.090	45.1	4.7	0.047 High carbohydrate in diet. High calories per kilogram
62	J. M.	7	5,900	Normal	0.204	0.063	30.8 <sup>a</sup>	4.2	0.049
126	W. H. 4	14	6,467	Nonhomogeneous	0.195	0.130	66.7	5.5	0.035 Recovering from rickets. Taking cod liver oil

49	G. H.	7.5	5,660	Constipated	0.193	0.090	46.8	9.5	0.023	Top milk formula. Cod liver oil. Very high calories per kilogram
119	W. H. 5	14	6,536	Nonhomogeneous	0.191	0.115	60.5	3.9	0.049	Recovering from rickets. Cod liver oil just discontinued
101	J. K. 2	16	4,720	Soft, normal	0.180	0.083	45.9	3.5	0.052	Underfed
67	M. R.	6	6,860	Constipated	0.179	0.042	23.6	3.4	0.053	
81	R. B.	15	9,075	Normal	0.177	0.097	55.0	3.4	0.052	15 cc. milk of magnesia
66	P. S. 3	10	6,560	Soft, normal	0.167	0.064	38.2	3.6	0.046	High proportion carbohydrate in diet
96	R. L. 1	12	10,400	Normal	0.152	0.069	45.3	2.9	0.053	Very low calories per kilogram
92	L. B.	3.5	3,535	Nonhomogeneous	0.148	0.051	34.6	3.5	0.042	Sweetened condensed milk. Very high proportion carbohydrate
83	A. P.	6	6,540	Soft, normal	0.136	0.070	51.7	2.3	0.059	Very low calories per kilogram
50	B. P.	10	9,574	Normal	0.088	0.014	15.2	3.8	0.023	Top milk formula. Low calories per kilogram
68	M. M.	11	11,490	Normal	0.087	0.013	16.0	2.1	0.043	Much underfed. Rather high proportion carbohydrate in diet
94	E. W.	5	3,770	Soft, normal	0.069	-0.064	0.0	0.9	0.077	Malted milk. Extremely high proportion carbohydrate in diet

values are expressed as grams per kilogram of body weight. Only in this way could the intake for infants of widely differing weights be correlated. A study of the values for total intake and absorption of calcium, weight not being considered, showed no consistent variation in absorption as related to intake.

*Calcium Absorption and Its Relation to Calcium Intake.*—The intake of calcium oxid per kilogram ranged from 0.13 to 0.30 gm., with an average of 0.22 gm. in twenty-six of the thirty cases studied. Of the remaining four cases, in one the intake of calcium oxid was extremely high, and in the others it was very low. In twenty instances the intake of calcium oxid was more than 0.19 gm.

TABLE 2.  
*Relation of Calcium Absorption to Calcium Intake.*

CaO Intake per Kg.	No. of Cases	Absorption of CaO Over 0.09 Gm. per Kg.	Absorption of CaO, 0.06 to 0.09 Gm. per Kg.	Absorption of CaO Under 0.06 Gm. per Kg.
Over 0.19 gm.	20	15	3	2
Under 0.19 gm.	10	1	4	5
Under 0.10 gm.	3	0	0	3

The relation of the calcium absorption to the calcium intake is summarized in Table 2. It is here seen that the best absorption occurred when the intake exceeded 0.19 gm. per kilogram, and that when the intake was less than 0.10 gm. the absorption was very poor. In the one case with exceptionally high intake, the absorption was only 0.099 gm. per kilogram, which was lower than in many cases where the intake was less.

The average absorption of calcium oxid in all the cases in which the intake exceeded 0.10 gm. per kilogram, was 0.089 gm. per kilogram. This value is higher than that found for breast fed infants. With five breast fed infants, having an intake of calcium oxid ranging from 0.045 to 0.097 gm. per kilogram and averaging 0.081 gm., we found an absorption of from 0.035 to 0.071 gm. per kilogram averaging 0.054 gm. In the much quoted case reported by Blauberg<sup>3</sup> the intake of calcium oxid was 0.041 gm. per kilogram and the absorption was 0.031 gm. per kilogram.

3 Blauberg: Ztschr. f. Biol. 40: 36, 1900.

*Relation of Calcium Absorption to Fat Intake.*—In twelve of the sixteen cases in Table 1 in which the absorption of calcium oxid exceeded 0.09 gm. per kilogram, the intake of fat exceeded 4.2 gm. per kilogram. In the other four cases in which the absorption of calcium oxid exceeded 0.09 gm. per kilogram the fat intake was less than 4.2 gm. per kilogram, but other factors were present which may have influenced the calcium absorption. One child had been receiving cod liver oil up to the time of the observation; another child had received one-half ounce of milk of magnesia; the other two children were taking a dried milk formula, in which the calcium may be present in a more easily assimilated form as a result of the treatment to which the milk has been subjected during the process of drying. In only four of the fourteen cases in which the absorption of calcium oxid was less than 0.09 gm. per kilogram was the fat intake as great as 4.2 gm. per kilogram.

In two-thirds of the cases in the table the food contained from 0.045 to 0.060 gm. of calcium oxid for every gram of fat. When the calcium oxid per gram of fat in the intake was less than 0.045 gm. per kilogram, the only high absorption of calcium oxid occurred when cod liver oil was being taken. This optimum ratio of fat and calcium intake existed in some cases when the intake of both calcium and fat was too low. It has been noted that the best calcium absorption took place when the intake of fat was 4.2 gm. or more per kilogram. The lowest value for calcium oxid intake per kilogram which would maintain the optimum ratio of calcium oxid to this amount of fat in the intake therefore would be 0.19 gm. per kilogram, a value already noted as the amount necessary to secure good absorption.

*Percentage Absorption of Calcium.*—From 35 to 55 per cent. of the calcium intake was absorbed in eighteen of the thirty cases. The only higher values for percentage absorption were noted twice with a child who was recovering from rickets and who had been receiving cod liver oil for a long period. A higher percentage of the calcium intake is absorbed by breast fed infants. In our cases the average for breast fed infants was 66.7 per cent.; in Blauberg's case the absorption was 75.6 per cent.

*Calcium Requirement.*—The average absorption of calcium oxid found for five breast fed infants was 0.054 gm. per kilogram. Since,

according to our observations, infants taking modifications of cow's milk absorbed on the average only about 45 per cent. of the calcium intake, it is necessary to provide for them a minimum intake of about 0.130 gm. of calcium oxid per kilogram to insure even the low average absorption of breast fed infants; there would, therefore, seem to be danger in an intake lower than this. On the other hand, there appears to be no advantage in an intake greater than 0.30 gm. per kilogram. Hoobler<sup>4</sup> says that anything less than 1.0 or 1.5 gm. of calcium oxid daily should be considered a calcium poor intake for infants taking modifications of cow's milk. His requirement, calculated for an infant of seven kilograms body weight, would be from 0.143 to 0.215 gm. of calcium oxid per kilogram of body weight. This range includes our above noted value, 0.19 gm., the intake of calcium oxid per kilogram found to be necessary to secure good absorption.

*Other Factors in Relation to Calcium Absorption.*—The values presented in Table 1 show that there was no definite variation in calcium absorption per kilogram according to the age or weight of the child.

There was also no definite relation between the type of stool and the amount of calcium oxid absorbed per kilogram or the percentage of the calcium intake absorbed.

Table 3 shows for the cases considered in Table 1 the calcium excretion in the stools in relation to the actual calcium and the fat intake; also the relation of the calcium excretion to the excretion of fat and of soap in the stools.

*Calcium Excretion and Its Relation to Calcium Intake and to Fat Intake.*—With the exception of two unusually high values the excretion of calcium in the stools ranged from 0.34 to 1.06 gm., with an average of 0.70 gm. As a rule, the higher the calcium intake the higher the calcium excretion; the lower the intake, the lower the excretion. In general, the fat intake and the calcium intake were parallel, so that a high calcium excretion occurring with a high fat intake could be accounted for by a high calcium intake. When a high fat intake accompanied a low calcium intake, the excretion of calcium was low, except in one case. Hence, there is little evidence that there is any constant relation between the amount of the fat intake and the amount of calcium excreted in the stools.

4. Hoobler: Am. J. Dis. Child. 2: 107, 1911.

TABLE 3.

*Calcium Excretion of Healthy Infants in Relation to Actual Calcium and Fat Intake.*

No.	Case	Stools	CaO Intake, Gm. Daily	Fat Intake, Gm. Daily	CaO in Stools, Gm. Daily	Fat in Stools, Gm. Daily	Fat as Soap in Stools, Gm. Daily	CaO Possibly Held as Soap, Percentage of	
								CaO in Stools	CaO Intake
113	J. S. 1	Nonhomogeneous	1.98	26.6	1.58	1.53	0.26	1.6	1.3
158	H. B.	Normal	1.84	34.7	1.54	4.93	3.90	25.3	21.2
71	P. D. 2	Constipated	1.20	22.6	1.06	3.34	2.64	24.9	22.0
65	P. D. 3	Constipated	1.39	24.0	1.06	2.95	2.05	19.3	14.7
54	R. C.	Normal	1.78	32.8	1.00	2.70	1.97	19.7	11.1
67	M. R.	Constipated	1.23	23.4	0.94	2.79	2.25	24.0	18.3
62	J. M.	Normal	1.20	24.4	0.89	1.14	1.01	11.4	9.6
57	M. S. 2	Normal	1.63	29.4	0.87	3.36	2.42	27.8	14.9
96	R. L. 1	Normal	1.58	30.0	0.86	2.04	1.47	17.0	9.3
58	J. I.	Normal	1.56	28.6	0.84	1.30	1.11	13.2	7.1
68	M. M.	Normal	1.00	23.3	0.84	1.24	0.96	11.5	9.6
70	P. D. 1	Constipated	1.22	22.9	0.78	2.21	1.67	22.3	13.7
63	F. N. 2	Soft, normal	1.29	24.1	0.75	5.09	3.68	49.1	28.6
81	R. B.	Normal	1.60	31.0	0.72	3.91	3.48	48.3	21.7
50	B. P.	Normal	0.84	36.5	0.71	2.91	2.01	29.2	24.7
82	P. L.	Nonhomogeneous	1.19	15.7	0.69	1.73	1.12*	16.3*	9.4*
66	P. S. 3	Soft, normal	1.10	23.7	0.68	0.85	0.30	4.4	2.7
97	B. W. 1	Soft, normal	1.36	27.6	0.67	2.54	1.81	27.6	13.3
60	F. N. 1	Constipated	1.22	26.2	0.67	4.12	3.31	49.4	27.1
86	J. M.	Soft, normal	1.02	13.4	0.66	2.08	1.67	25.3	16.4
72	A. K.	Constipated	1.16	22.1	0.64	3.04	2.22	34.7	19.1
49	G. H.	Constipated	1.09	48.3	0.58	2.98	2.33	40.2	21.4
166	A. A.	Nonhomogeneous	0.78	11.4	0.56	2.46	0.04	0.7	0.4
94	E. W.	Soft, normal	0.26	3.4	0.50	1.00	0.75	15.0	29.0
119	W. H. 5	Nonhomogeneous	1.24	25.2	0.49	2.64	0.78*	15.9*	6.3*
101	J. K. 2	Soft, normal	0.85	16.5	0.46	0.66	0.46	10.0	5.4
83	A. P.	Soft, normal	0.89	15.1	0.43	0.65	0.50	11.6	5.6
126	W. H. 4	Nonhomogeneous	1.26	35.8	0.42	3.35	1.69*	40.2*	13.4*
85	C. M.	Nonhomogeneous	0.79	14.4	0.39	1.64	1.05*	26.9*	13.3*
92	L. B.	Nonhomogeneous	0.52	12.5	0.34	2.06	1.38*	40.7*	26.6*

\* Value possibly too high. Stools acid.

*Relation of Calcium Excretion to Excretion of Total Fat and of Fat as Soap.*—There was no constant relation between the excretion of total fat and the excretion of calcium in the stools. In order to study the relation between the excretion of calcium and that of fat as soap,

the findings shown in Table 3 were averaged by groups according to the type of stool, since the average soap excretion varies definitely with the type of stool. These averages (Table 4) show that the calcium excretion was more closely related to the calcium intake than it was to the excretion of soap. In the constipated stools there was a greater soap content than in the normal stools, but a smaller content of calcium, corresponding to a smaller calcium intake. The group of soft, normal stools had a much greater soap content than that of the nonhomogeneous stools, but here, again, a somewhat lower calcium content corresponded to a somewhat lower calcium intake.

There is a prevalent opinion that in soapy stools an excessive loss of calcium occurs, which may take place either as calcium bound to the fat in the form of insoluble soaps or as calcium phosphate. To

TABLE 4.

*Relation of Average Calcium Excretion to the Average Soap Excretion.*

Stools	No. of Cases	CaO Intake, Gm. Daily	CaO in Stools, Gm. Daily	Fat as Soap in Stools, Gm. Daily	CaO Possibly Held as Soap	CaO not Held as Soap	CaO Possibly Held as Soap, Percentage of	
							CaO in Stools	CaO Intake
Constipated.....	7	1.22	0.82	2.35	0.24	0.58	30.9	19.5
Normal.....	9	1.45	0.92	2.04	0.20	0.72	22.6	14.4
Soft, normal.....	7	0.97	0.59	1.31	0.13	0.46	20.4	14.4
Nonhomogeneous.....	7	1.11	0.64	0.90	0.09	0.59	20.3	10.1

show to what extent calcium was lost as insoluble soaps, values have been included in Table 3 giving the percentage of the total calcium *excretion* which could possibly be held in the form of soap, and also the percentage of the calcium *intake* which could be lost in this way. The amount of calcium that could be bound as soap is approximately one-tenth the amount of soap in the stool, since the combining ratio of calcium to the higher fatty acids is about one to ten. (Combining weight of calcium oxid is 28; stearic acid, 284.) The calcium bound as soap was never found to be as much as five-tenths and seldom more than three-tenths of the total calcium excretion. In all the cases in which the calcium lost as soap was more than three-tenths of the total calcium excretion the *total* loss of calcium was not excessive. In

no case was the calcium lost as soap equal to three-tenths of the calcium *intake*.

The average values according to type of stool, included in Table 4, show that when the stools were constipated a greater proportion of both the calcium excretion and the calcium intake was lost as soap, but that the total loss of calcium and also the calcium intake averaged distinctly less for this group than for the group of normal stools.

Table 4 also includes average values to show to what extent calcium could be lost as phosphate in the different types of stools. These values were obtained by subtracting from the average total calcium excretion the amount of calcium which could be held as soap, that is, one-tenth of the value for soap excretion. The remaining amount of calcium in the stools is the maximum that could be held as phosphate. Thus, it is shown that the amount of calcium that could be held as phosphate was greatest in the group of normal stools, which had lower soap content than did the constipated stools. In fact, the average value for the calcium not held as soap for the group of constipated stools with the highest soap content was practically the same as that for the group of nonhomogeneous stools with the lowest soap content. These observations seem to establish quite definitely that in soapy stools there is not an increased excretion of calcium phosphate. The excretion of calcium not held as soap was influenced more by the amount of calcium intake than by any other factor.

#### *Rachitic Infants.*

The second group of infants considered were suffering from rickets. Several of them also had tetany, either mild or severe. None of these children had diarrheal stools. Table 5 shows the absorption of calcium per kilogram and its relation to the calcium and the fat intake.

*Calcium Absorption.*—The intake of calcium oxid per kilogram of body weight was, in general, lower and the range was narrower than was the case with normal infants. In the majority of the cases the intake was less than 0.19 gm. per kilogram, which we have noted as the minimum to insure good absorption by healthy infants. The absorption of calcium oxid per kilogram by the rachitic infants as a

TABLE 5.  
*Absorption of Calcium by Rachitic Infants.*

No.	Case	Age in Mos.	Weight in Gm.	Stools	Intake of CaO Absorbed, Gm. per Kg.	Per Cent. Intake of CaO Absorbed	Intake of Fat, Gm. per Kg.	Grams of CaO Intake per Gm. Fat Intake	Remarks
390	J. D. 4	5	3,901	Soft, normal	0.249	0.077	30.9	6.7	Mild tetany. Cod liver oil for about one week
401	E. C. 1	11	6,724	Nonhomogeneous	0.247	-0.013	0.0	5.0	0.049
64	L. R. 2	12	3,883	Soft, normal	0.242	0.136	56.2	6.3	Cod liver oil
402	L. R. 1	9.5	3,454	Loose	0.240	0.055	22.9	4.6	0.053
388	J. D. 1	5	3,845	Loose	0.235	0.065	27.6	3.9	0.060 Tetany. One very severe glottis spasm
389	J. D. 3	5	3,976	Loose	0.228	0.084	36.8	5.1	0.045 Cod liver oil for few days before observation
397	L.E.	8.5	7,395	Nonhomogeneous	0.210	0.029	13.8	4.7	0.045 Mild tetany, marked rickets
61	E. C. 2	14	7,789	Constipated	0.193	0.032	16.6	3.3	0.059 Rather high proportion carbohydrate in diet
400	H. S.	9	7,825	Nonhomogeneous	0.184	0.049	26.6	2.5	0.073 Protein milk. Recently changed from breast. Severe tetany
394	J. T. 1	9	6,734	Constipated	0.183	0.012	6.6	3.7	0.049
74	E. C. 3	15	7,788	Soft, normal	0.181	-0.008	0.0	2.6	0.067 Rather low calories per kilogram
395	J. T. 2	9	6,827	Constipated	0.177	0.036	20.3	3.7	0.048 Injection saline first day
398	B. M. 1	7	5,646	Nonhomogeneous	0.165	0.063	38.1	3.8	0.044 Mild rickets, tetany
391	P. L. 1	10	9,612	Constipated	0.158	0.055	34.7	3.3	0.048 Recently changed from breast milk. High carbohydrate. Mild rickets
393	P. L. 3	10	9,560	Normal	0.153	0.019	12.4	3.2	0.049 High carbohydrate. Mild rickets
387	D. W. 3	5	5,733	Nonhomogeneous	0.153	0.038	24.8	4.0	0.038 Cod liver oil. Magnesium sulphate injection. Convulsions
392	P. L. 2	10	9,574	Constipated	0.152	0.057	37.5	3.2	0.048 Mild rickets. Injection of magnesium sulphate

386	D. W. 1	5	5,815	Nonhomogeneous	0.148	0.055	37.2	3.1	0.048	Frequent and severe convulsions. Recently changed from breast milk
155	D. W. 2	5	5,816	Nonhomogeneous	0.145	0.065	44.8	3.0	0.049	Low calories per kilogram. Tetany with convulsions
154	W. H. 1	12	7,048	Nonhomogeneous	0.133	-0.017	0.0	2.5	0.054	Rather low calories per kilogram
399	T. W.	9	5,144	Nonhomogeneous	0.124	0.020	16.1	2.4	0.051	Mild tetany
117	W. H. 2	12	7,140	Normal	0.117	0.020	17.1	3.6	0.033	Cod liver oil for two days before observation.
396	G. S.	9.5	7,445	Nonhomogeneous	0.073	0.033	45.2	3.0	0.024	Low calories per kilogram
										Recently changed from breast milk. Cod liver oil. Marked rickets. Mild tetany

group was extremely poor. In only one observation was there good absorption, that is, more than 0.09 gm., the average found for normal infants. In only six of the twenty-three observations was the absorption as much as 0.06 gm. per kilogram. In the cases where there was fair absorption of calcium oxid, the children either had been taking cod liver oil or were at the time suffering from severe tetany. In the one instance in which the absorption was high the child had been receiving cod liver oil for two and one-half months. The two next highest values were found with a child who had been taking cod liver oil for about one week. In these three instances the fat intake per kilogram was the highest shown in the table and was accompanied by a high intake of calcium oxid. Although several children showing a very low absorption were at the time of observation taking cod liver oil, none had received it for more than a few days. Of five observations on children suffering from tetany with convulsions, in only one instance was the absorption of calcium oxid less than 0.05 gm. per kilogram, which is a value higher than the average for rachitic infants.

The average absorption for the group of rachitic infants was 0.042 gm. per kilogram, which is less than one-half that found for the normal infants.

The poor absorption of calcium in rickets is strikingly shown by the values for percentage absorption. In fifteen of the twenty-three cases the percentage absorption was lower than the usual range for normal children.

The intake of fat per kilogram as well as that of calcium per kilogram was, on the whole, lower with the rachitic than with the normal children. The relation of the calcium intake to the fat intake was in most cases similar to that of the normal group.

Table 6 gives for the rachitic children the calcium excretion in the stools together with the calcium and the fat intake. In the few cases in which fat values in the stools were obtained, there is shown the relation of the calcium excretion to that of total fat and fat as soap.

*Calcium Excretion.*—The range in calcium excretion was wider with rachitic than with normal children. There were seven values exceeding 1.0 gm. and the average, 0.86 gm., was higher than the normal average, 0.70 gm. In general, the excretion of calcium was related

to the calcium intake, but not to the fat intake. In the few cases in which fat determinations in the stools were made the calcium excretion was found to be entirely unrelated to the amount of either total

TABLE 6.  
*Calcium Excretion by Rachitic Infants.*

No.	Case	Stools	CaO Intake, Gm. Daily	Fat Intake, Gm. Daily	CaO in Stools, Gm. Daily	Fat in Stools, Gm. Daily	Fat as Soap in Stools, Gm. Daily	CaO Possibly Held as Soap, Percentage of	
								CaO in Stools	CaO In- take
401	E. C. 1	Nonhomogeneous	1.66	33.9	1.75	..	..	..	..
74	E. C. 3	Soft, normal	1.41	21.1	1.47	2.71	1.76	12.0	12.5
397	L. E.	Nonhomogeneous	1.56	34.9	1.34	..	..	..	..
393	P. L. 3	Normal	1.46	30.1	1.28	..	..	..	..
61	E. C. 2	Constipated	1.50	25.5	1.25	1.71	1.12	9.0	7.5
394	J. T. 1	Constipated	1.23	27.1	1.16	..	..	..	..
154	W. H. 1	Nonhomogeneous	0.94	17.4	1.06	0.91	0.49	4.7	5.2
391	P. L. 1	Constipated	1.52	31.4	0.99	..	..	..	..
395	J. T. 2	Constipated	1.21	25.0	0.97	..	..	..	..
400	H. S.	Nonhomogeneous	1.44	19.7	0.93	..	..	..	..
392	P. L. 2	Constipated	1.46	30.2	0.91	..	..	..	..
117	W. H. 2	Normal	0.84	25.4	0.69	2.24	..	..	..
390	J. D. 4	Soft, normal	0.97	26.2	0.67	..	..	..	..
388	J. D. 1	Loose	0.90	15.1	0.66	..	..	..	..
387	D. W. 3	Nonhomogeneous	0.88	23.0	0.66	..	..	..	..
402	L. R. 1	Loose	0.83	15.7	0.64	..	..	..	..
389	J. D. 3	Loose	0.90	20.1	0.57	..	..	..	..
398	B. M. 1	Nonhomogeneous	0.93	21.1	0.57	..	..	..	..
386	D. W. 1	Nonhomogeneous	0.86	17.9	0.54	..	..	..	..
399	T. W.	Nonhomogeneous	0.64	12.6	0.53	..	..	..	..
155	D. W. 2	Nonhomogeneous	0.84	17.2	0.46	3.73	2.06*	44.7*	24.5*
64	L. R. 2	Soft, normal	0.94	24.6	0.41	3.00	1.21*	29.5*	12.9*
396	G. S.	Nonhomogeneous	0.55	22.6	0.30	..	..	..	..

\* Value possibly too high. Stools acid.

fat or fat as soap in the stools. In two instances there was a very large fat excretion in the stools and a very small calcium excretion; in three others there was a high calcium excretion, but only a small part of this excretion was in the form of soap.

*Infants Suffering from Diarrhea.*

The third group of infants considered were suffering from diarrhea. Some of them were also rachitic, but are placed in this group because the diarrheal condition is the chief factor in determining the composition of the stools. Table 7 gives the absorption of calcium oxid per kilogram and its relation to the calcium and fat intake for these infants.

*Calcium Absorption.*—The range in intake of calcium per kilogram was very wide, and there were more high values than were found with normal infants. This was due largely to the fact that these infants were much under size. The absorption of calcium oxid was generally low. Only five of the nineteen values exceeded 0.06 gm. per kilogram; only two exceeded 0.09 gm. per kilogram. One of these two children had been receiving cod liver oil for about one week, and the other had a very high intake of calcium. The third highest absorption was in the case of a child who had been receiving cod liver oil just before the period of observation.

The average absorption for this group of infants was 0.046 gm. of calcium per kilogram, about one-half that found for normal children.

The percentage absorption was also much below normal. Only four of the nineteen values fell within the normal range.

The fat intake per kilogram varied widely and bore no constant relation to the calcium absorption. Successive observations on two children, V. C. and E. R., showed that the calcium absorption increased as the diarrhea improved. Both these children were taking cod liver oil.

Table 8 shows the calcium excretion and its relation to the calcium and the fat intake and to the excretion of total fat and of fat as soap in the stools of infants with diarrhea.

*Calcium Excretion.*—Both the calcium excretion and the calcium intake showed a wide range. The higher excretion usually occurred with the higher intake. The average excretion of calcium oxid was 0.86 gm. As in the other groups there was no definite relation between the fat intake and the calcium excretion. In some instances when the fat intake was high the calcium intake was also high, and the excretion of calcium higher than when the intake of calcium was lower. In this group, as in the others studied, there was no relation

TABLE 7.  
*Absorption of Calcium by Infants Suffering from Diarrhea.*

No.	Case	Age in Mo.	Weight in Gm.	Stools	Intake of CaO Ab-sorbed, Gm. per Kg.	Per Cent. of CaO Intake absorbed	Grams of CaO or Fat, per Gm. per Kg.	Remarks
135	F. H. 4	11	4,838	Diarrheal	0.412	0.113	27.6	4.1 0.100
109	S. J.	9	4,922	Severely diarrheal	0.362	0.063	17.4	5.9 0.061 Evaporated milk formula. Very large fermentative stools
132	F. H. 1	10	4,442	Severely diarrheal	0.348	0.059	16.9	7.3 0.048
136	F. H. 2	10	4,660	Diarrheal	0.332	0.043	13.0	7.2 0.046
175	I. G.	2.5	2,930	Diarrheal	0.304	0.021	6.7	5.0 0.061 Evaporated milk formula. Very large fermentative stools
106	V. C. 3	5	4,419	Diarrheal	0.282	0.138	49.2	6.8 0.041 Tetany, rickets, taking cod liver oil
121	V. C. 2	5	4,575	Severely diarrheal	0.248	0.059	23.9	5.2 0.048
120	V. C. 1	5	4,666	Severely diarrheal	0.234	0.062	26.6	5.1 0.048
143	F. S.	6	4,147	Mildly diarrheal	0.234	0.000	0.0	4.5 0.052 Considerable barley flour in formula
139	J. D. 2	5	3,930	Diarrheal	0.217	0.038	17.7	4.4 0.050 Rickets, taking cod liver oil.
152	M. R.	4	3,001	Diarrheal	0.170	0.023	13.7	1.8 0.095 High proportion carbohydrate in diet
144	V. R. 2	3	6,357	Mildly diarrheal	0.154	0.038	24.5	2.9 0.052 Recently changed from breast milk
127	D. W. 4	5	5,724	Diarrheal	0.147	0.053	35.7	3.9 0.038 Rickets, tetany, taking cod liver oil
123	E. R. 3	11	7,367	Diarrheal	0.136	0.058	43.0	3.2 0.043 Rickets, tetany, taking cod liver oil. Low
147	W. H. 3	14	6,734	Diarrheal	0.134	0.071	53.3	2.7 0.049 Rickets, tetany, taking cod liver oil just discontinued
111	E. R. 2	10	7,436	Diarrheal	0.128	0.030	23.2	3.6 0.035 Rickets, tetany, taking cod liver oil. Low
131	E. R. 1	10	7,540	Diarrheal	0.123	0.000	0.0	2.2 0.055 Rickets, tetany, had castor oil. High proportion carbohydrate in diet
164	B. S. 1	12	8,105	Diarrheal	0.107	0.010	8.3	1.9 0.063 Rickets, tetany. Rather high carbohydrate in diet
176	B. S. 2	13	7,708	Severely diarrheal	0.095	0.004	4.1	1.5 0.063 Rickets, tetany. Rather high carbohydrate in diet. Excessively large stool

between the calcium excretion and the total fat excretion in the stools. Since the excretion of fat as soap in these acid stools was very low, evidently there was no relation between the excretion of calcium and that of fat as soap.

TABLE 8.  
*Calcium Excretion by Infants Suffering from Diarrhea.*

No.	Case	Stools	CaO	Fat	CaO	Fat	CaO Possibly	
			Intake, Gm. Daily	Intake, Gm. Daily	in Stools, Gm. Daily	in Stools, Gm. Daily	as Soap in Stools, Gm. Daily	Held as Soap, Percentage of
								CaO in Stools
109	S. J.	Severely diarrheal	1.78	29.1	1.47	14.54	1.29*	8.8* 7.3*
135	F. H. 4	Diarrheal	1.99	20.0	1.44	1.77	0.22*	1.5* 1.1*
136	F. H. 2	Diarrheal	1.54	33.6	1.34	4.61	0.87*	6.5* 5.6*
132	F. H. 1	Severely diarrheal	1.54	32.4	1.28	6.91	1.06*	8.3* 6.9*
143	F. S.	Mildly diarrheal	0.97	18.7	0.98	4.20	1.19*	12.2* 12.3*
131	E. R. 1	Diarrheal	0.93	16.8	0.94	3.35	0.00	0.0 0.0
164	B. S. 1	Diarrheal	0.97	15.5	0.89	3.07	0.00	0.0 0.0
121	V. C. 2	Severely diarrheal	1.13	23.6	0.86	10.38	0.34*	4.0* 3.0*
175	I. G.	Diarrheal	0.89	14.7	0.83	11.62	0.86*	10.4* 9.7*
120	V. C. 1	Severely diarrheal	1.09	23.6	0.80	5.77	0.00	0.0 0.0
144	V. R. 2	Mildly diarrheal	0.98	18.7	0.74	1.88	0.58*	7.8* 5.9*
111	E. R. 2	Diarrheal	0.95	27.1	0.73	3.76	0.00	0.0 0.0
139	J. D. 2	Diarrheal	0.85	17.0	0.70	6.01	0.82*	11.7* 9.7*
176	B. S. 2	Severely diarrheal	0.73	11.6	0.70	10.08	0.00	0.0 0.0
106	V. C. 3	Diarrheal	1.24	30.2	0.63	6.73	0.00	0.0 0.0
123	E. R. 3	Diarrheal	1.00	23.4	0.57	4.97	0.00	0.0 0.0
127	D. W. 4	Diarrheal	0.84	22.2	0.54	5.38	0.82*	15.2* 9.8*
152	M. R.	Diarrheal	0.51	5.4	0.44	3.61	0.13*	2.8* 2.6*
147	W. H. 3	Diarrheal	0.90	18.4	0.42	2.10	0.57*	13.7* 6.4*

\* Value possibly too high. Stools acid.

#### *Proportion of Calcium and of Soap in the Stools of Infants.*

Table 9 gives the average proportion of calcium in various types of stools of infants and its relation to the other constituents of the stools and to the calcium intake. There are given values not only for stools of infants taking modifications of cow's milk, but also for a number of stools of breast fed infants.

The stools of infants taking modifications of cow's milk which contained the smallest proportion of water, that is, the constipated and the normal stools, showed on the average the higher calcium percentage of total solids. With increased proportion of water the calcium percentage of total solids became less. The total calcium excretion

TABLE 9.  
*Average Proportions of Calcium and of Soap in Stools of Infants.*

Condition of Infants	Food	Stools	No. of Cases	CaO Intake, Gm. Daily	CaO Percentage of Total Solids	CaO Percentage of Total Solids	Fat as Soap, Percentage of Total Solids
Normal	Cow's milk	Constipated	13	1.21	11.1	49.6	30.0
Normal	Cow's milk	Normal	19	1.23	11.8	47.5	24.5
Nearly normal	Cow's milk	Soft, normal	7	1.06	10.0	46.3	17.7
Mostly delicate	Cow's milk	Nonhomogeneous	13	1.02	7.9	40.9	13.6*
Sick	Cow's milk	Diarrheal	13	1.05	6.3	32.5	2.8*
Sick	Cow's milk	Severely diarrheal	6	1.12	4.4	25.2	2.8*
Rachitic	Cow's milk	Constipated or normal	9	1.37	12.7	44.7	15.7**†
Rachitic	Cow's milk	Soft, normal or non-homogeneous	14	0.94	8.1	39.1	15.5**‡
Normal	Breast milk	Normal	3	(0.38)§	3.4	37.4	23.4*
Normal	Breast milk	Nearly normal, partly green	12	(0.38)§	4.3	42.9	12.9*
Normal	Breast milk	Green, with mucus	5	(0.38)§	3.0	39.6	4.2*
Sick	Breast milk	Diarrheal	4	0.22	1.8	18.4	4.6*
M. 1, normal	Top milk, cream dilution	Normal	1	0.27	2.1	28.0	50.6
E. W., normal	Malted milk	Soft, normal	1	0.26	7.5	40.6	11.0

\* Value possibly too high. Stools acid.

† Only two values included in this average.

‡ Only three values included in this average.

§ Average of five values for normal children.

was not thereby diminished, however, but rather increased, since the daily amount of total solids was greatly increased with the higher water content. The calcium percentage of total solids appeared to be more closely related to the looseness of the stools than to the amount of calcium intake, since there was but little variation in average intake for the different groups.

The calcium percentage of total solids of the constipated or normal stools of rachitic infants was higher than that of the corresponding type of stools of normal infants. It is not evident whether this increased percentage was due to the condition of rickets or to the higher average intake.

The average values for calcium percentage of total *salts* also were less as the stools became more watery. In other words, the proportion of salts not calcium, that is, the soluble salts, increased with the water content of the stools.

In stools of rachitic infants, although the total salts were increased over the normal, the calcium formed a smaller percentage of the total salts than in the corresponding stools of normal children. This shows that the other salts were increased more than was the calcium.

The proportion of the total solids which was soap in the stools of infants taking modifications of cow's milk was closely related to the water content, diminishing strikingly as the water in the stools increased. In the looser types of stools the decrease in soap percentage with increase in water content was accompanied by a similar though less marked decrease in the proportion of calcium. This association was not found in the constipated and the normal stools. The normal stools showed a lower proportion of fat as soap, with a slightly higher percentage of calcium, than did the constipated stools.

An entirely different range of values was seen with breast fed infants. With a much lower intake of calcium, the calcium percentage of total solids in the stools was much lower than when the food was modifications of cow's milk. The diarrheal stools of breast fed infants showed a much lower calcium percentage of both total solids and total salts than did the other types of stools. The other groups of the stools of breast fed infants did not differ much from one another in water content and varied little in the calcium percentage of total solids or of total salts. The calcium formed a somewhat smaller part of the total salts than in the stools of artificially fed infants.

The fat as soap, as well as the calcium oxid, formed a smaller proportion of the total solids in the stools of breast fed infants than in those of infants taking cow's milk, and was less in the diarrheal than in the normal stools. In the three groups of stools of breast fed infants which were normal as to water content, the proportion of soap

was lower the less normal the appearance of the stool, while the calcium percentage of total solids varied but little.

The proportion of calcium in the stools in two instances of unusual feeding, with extremely low calcium intake, are included in Table 9. In the first instance, in which the food was a cream dilution, the calcium percentage of both total solids and total salts was very low, even lower than that found with breast fed infants. This stool had a very high percentage of soap, due to the administration of a large amount of milk of magnesium. In the other instance, in which the child received malted milk, the calcium percentage of total solids and of total salts was somewhat lower than that found for the same type of stool when the food was a modification of cow's milk.

The following summary gives answers, based on our observations, to such of the questions stated in the introduction as apply to infants taking modifications of cow's milk.

#### SUMMARY.

1. The average absorption of calcium oxid by healthy infants taking modifications of cow's milk was 0.09 gm. per kilogram of body weight. Since the average absorption of calcium oxid by breast fed infants was about 0.06 gm. per kilogram, it may be assumed that 0.06 gm. per kilogram is the minimum normal absorption by infants taking modifications of cow's milk.

The daily total excretion of calcium oxid in the stools ranged from 0.34 to 1.06 gm., averaging 0.70 gm.

2. The excretion and the absorption of calcium were, in general, dependent on the amount of calcium intake, from 35 to 55 per cent. of the intake being absorbed.

To insure the average absorption of 0.09 gm. of calcium oxid per kilogram, the intake of calcium oxid should be at least 0.19 gm. per kilogram; to insure an absorption equal to the average found for breast fed infants the intake of calcium oxid should be at least 0.13 gm. per kilogram.

The best absorption of calcium was obtained when the calcium intake bore a definite relation to the fat intake, that is, when the food contained from 0.045 to 0.060 gm. of calcium oxid for every gram of

fat and when at the same time the fat intake was ample, not less than 4.0 gm. per kilogram.

3. An excessive calcium intake apparently did not increase the calcium absorption, the excess being excreted. When the intake of calcium oxid was very low, less than 0.10 gm. per kilogram, the absorption of calcium oxid was less than the normal calcium requirement of the body.

4. The *total* absorption of calcium oxid varied in general with the weight of the child; the *per kilogram* absorption did not vary regularly with either the age or the weight.

5. The relation of calcium excretion to soap excretion was not constant. The excretion of *soap* was directly related to the type of stool, that is, to the water content and to the reaction of the stool. The excretion of *calcium* was closely related to the calcium intake. On the average, the normal and the constipated stools, with high soap content, were found when the intake of calcium was high and, therefore, they showed the higher excretion of calcium. However, constipated stools, which contained more soap than normal stools, had lower calcium content. Nonhomogeneous stools, with the lowest average soap content, showed the same content of calcium not held as soap as did the constipated stools, with the highest soap content.

6. The calcium that could be lost as soap was never a large proportion of the calcium intake. Even in the stools containing the most soap it was found to be less than three-tenths the calcium intake. The calcium lost as phosphate was shown not to be increased in soapy stools.

7. The calcium percentage of the total solids varied, as a rule, with the water content of the stools, diminishing as the water increased.

8. The calcium absorption was much lower when diarrhea was present. With an increased excretion of calcium in diarrheal stools, there was a marked decrease in soap excretion.

The calcium absorption by rachitic infants was much lower than that by healthy infants.

In the few cases in which observations were made on infants recovering from rickets, the calcium absorption was higher than the normal average. These infants had received cod liver oil for a considerable period.

9. The administration of cod liver oil regularly increased the absorption of calcium, unless diarrhea was present.

[Reprinted from the AMERICAN JOURNAL OF DISEASES OF CHILDREN, March, 1920,  
Vol. xix, No. 3, pp. 201-222.]

## CALCIUM METABOLISM OF INFANTS AND YOUNG CHILDREN, AND THE RELATION OF CALCIUM TO FAT EXCRETION IN THE STOOLS.

### PART II. CHILDREN TAKING A MIXED DIET.

By L. EMMETT HOLT, M.D., ANGELIA M. COURTNEY, AND HELEN L. FALES.

(*From the Laboratories of The Rockefeller Institute for Medical Research and the Babies' Hospital.*)

In a preceding paper on calcium metabolism were considered the absorption of calcium by infants taking modifications of cow's milk and the excretion of calcium in relation to the excretion of fat in their stools. In this paper we discuss the calcium metabolism of older children taking a mixed diet. As stated in the previous paper, by calcium *excretion* is meant the total amount of calcium lost in the stools; whether part of this has been absorbed and is subsequently excreted into the large intestine is not here considered. By calcium *absorption* is meant the difference between intake and the amount lost in the stools.

#### *Healthy Children.*

The first group considered includes children who were normal as to digestion. Tables 1 and 2 show the calcium absorption in relation to calcium and fat intake for normal children taking a mixed diet; Table 1 including the cases in which the intake of calcium oxid was high, and Table 2 those in which it was low. The values are expressed as grams per kilogram of body weight.

*Calcium Absorption and Its Relation to Calcium Intake.*--Excluding two unusually high values and one unusually low value, the intake of calcium oxid per kilo ranged from 0.043 to 0.178 gm., with an average of 0.108 gm. In only about one-half the cases was the intake more than 0.1 gm. per kilo. The children taking a mixed diet had, on the whole, a much lower intake of calcium oxid per kilo than those who were taking modifications of cow's milk.

TABLE I.  
*Absorption of Calcium by Healthy Children Taking a Mixed Diet, with an Intake of Calcium Oxid Exceeding 0.09 Gm. per Kilo.*

No.	Case	Age Yr. Mo.	Weight in Gm.	Stools	Intake of CaO Gm. per Kg.	CaO Ab- sorbed, Gm. per Kg.	Per- centage of CaO ab- sorbed	Intake of CaO Gm. per Kg.	Grains CaO Intake per Gm. Fat Intake	Remarks
321	F. S.	1 6	8,975	Constipated	0 244	0.147	60.4	6 8	0.036	Very high calories per kilogram
305	H. F. 1	1 0	7,136	Constipated	0.237	0.115	48.4	4 9	0.048	
307	H. F. 3	1 0	7,749	Constipated	0.178	0.018	10.1	4.7	0.038	
318	D. L.	1 2	8,264	Constipated	0.176	0.087	49.6	4.1	0.043	High calories per kilogram, high proportion carbohydrate
267	O. W. 1	2 6	9,988	Normal	0.175	0.081	46.3	3.8	0.046	
268	O. W. 2	2 7	10,280	Constipated	0.171	0.107	63.1	3 7	0.046	
364	O. W. 4	2 7	10,546	Normal	0.170	0.104	61.4	4.5	0.038	Skimmed milk with corn oil
379	H. F. 4	1 1	8,218	Normal	0.167	0.025	15.2	4.5	0.037	Nut butter
269	O. W. 5	2 8	10,714	Constipated	0.165	0.103	62.5	5.0	0.033	Cod liver oil
308	H. F. 5	1 1	8,455	Constipated	0.163	0.033	20.3	4.2	0.039	Fat-free milk, milk butter
380	H. F. 6	1 2	8,790	Normal	0.157	0.051	32.6	4.0	0.039	Fat-free milk, nut butter
225	C. A. 1	2 3	11,860	Normal	0.156	0.033	14.6	2.9	0.053	Whole milk only. Low total calories
312	R. K. 3	2 0	11,510	Normal	0.153	0.081	52.8	3.3	0.046	
233	F. W. 8	3 7	10,725	Normal	0.149	0.036	24.4	2.9	0.052	Whole milk and cereal. Low total calories
232	J. E.	1 11	10,438	Normal	0.145	0.050	34.4	2.7	0.054	Mostly whole milk. Low total calories
327	R. M.	4 0	12,650	Acid	0.144	0.057	39.4	4.0	0.036	Tuberculous peritonitis. Had cod liver oil
296	E. M. 1	2 3	13,000	Constipated	0.142	0.036	25.5	3.0	0.047	
311	R. K. 2	1 11	11,220	Constipated	0.141	0.036	25.3	2.9	0.052	
313	E. K.	2 0	12,540	Normal	0.140	0.090	64.3	3.0	0.046	
369	W. R. 6	4 9	15,950	Normal	0.137	0.091	66.6	4.0	0.035	Corn oil, considerable milk curd in diet
295	M. M.	5 6	14,777	Normal	0.131	0.016	12.4	2.6	0.050	
309	H. F. 7	1 2	9,170	Constipated	0.128	0.049	38.5	4.3	0.029	

289	M. C.	6	0	14.300	Normal	0.128	0.048	37.1	3.1	0.041
381	H. F. 8	1	3	9.425	Acid	0.124	0.075	60.7	4.6	0.026
270	A. W. 1	4	0	14.358	Constipated	0.123	0.061	50.0	2.7	0.046
317	K. P.	2	8	10.850	Normal	0.119	0.042	34.8	3.2	0.037
										Diabetes insipidus. Large proportion carbohydrate in diet
328	D. D.	1	8	11.300	Acid	0.119	0.041	34.3	3.7	0.032
277	R. L. 3	3	1	13.070	Acid	0.119	0.060	51.0	4.0	0.030
300	F. B. 1	3	8	15.334	Normal	0.118	0.059	50.2	3.2	0.053
320	J. O. 2	3	0	15.050	Constipated	0.117	0.064	54.6	2.6	0.046
291	R. K.	2	1	13.150	Normal	0.114	0.051	44.7	3.2	0.035
324	T. R.	4	0	15.395	Constipated	0.114	0.047	40.9	2.5	0.046
366	W. R. 3	4	7	15.700	Normal	0.112	0.037	32.9	4.9	0.023
367	W. R. 4	4	7	15.735	Normal	0.112	0.043	38.6	4.2	0.023
264	F. W. 9	3	7	11.105	Normal	0.109	0.025	23.1	3.9	0.028
372	F. W. 6	3	7	11.114	Normal	0.109	0.044	40.5	5.3	0.020
330	H. F.	4	6	16.130	Normal	0.109	0.024	22.2	2.4	0.046
373	F. W. 7	2	7	11.365	Normal	0.106	0.036	33.9	5.2	0.020
276	R. L. 2	3	0	12.879	Normal	0.105	0.012	11.0	3.7	0.028
287	W. W.	2	5	15.860	Normal	0.105	0.031	29.9	3.2	0.033
359	R. L. 4	3	1	13.478	Acid	0.103	0.054	52.5	4.7	0.022
326	E. A. 2	7	5	13.025	Constipated	0.096	0.041	42.4	2.6	0.037
290	D. R.	3	0	14.450	Constipated	0.096	0.025	25.7	3.2	0.030
322	M. J. 1	3	0	15.400	Constipated	0.093	0.058	62.2	3.1	0.030
271	A. W. 2	4	1	14.755	Normal	0.092	0.046	50.0	3.3	0.028
										Skinned milk, cod liver oil, rather high proportion carbohydrate

TABLE 2.

*Absorption of Calcium by Healthy Children Taking a Mixed Diet, with an Intake of 0.09 Gm. of Calcium Oxide per Kilo or Less.*

No.	Case	Age Yr. Mo.	Weight in Gm.	Stools	CaO Ab- sorbed, Gm. per Kg.	Intake of CaO, Gm. per Kg.	Per- centage of CaO absorbed	Grams CaO Intake per Gm. Fat Intake	Remarks
297	E. M. 2	2 4	13,100	Normal	0.090	0.026	28.8	2.5	0.036
301	F. B. 2	3 9	15,795	Acid	0.090	0.010	11.3	2.7	0.033
272	A. W. 3	4 1	15,334	Normal	0.089	0.050	56.5	3.4	0.026
302	F. B. 3	3 9	15,831	Normal	0.089	0.027	29.8	2.7	0.032
298	E. M. 3	2 6	13,744	Normal	0.088	0.028	31.4	2.5	0.036
234	A. L.	3 0	12,440	Normal	0.087	-0.015	0.0	1.8	0.050
299	E. M. 4	2 6	13,979	Normal	0.087	0.018	20.5	2.9	0.030
383	E. M. 7	2 8	14,040	Normal	0.087	0.004	4.9	2.6	0.032
274	A. W. 5	4 2	15,725	Normal	0.087	0.012	14.0	3.3	0.026
382	E. M. 5	2 6	14,175	Normal	0.086	0.020	23.8	2.8	0.031
254	E. M. 6	2 7	14,130	Normal	0.086	0.001	0.8	2.7	0.032
248	E. D.	2 6	10,305	Acid	0.084	0.018	22.1	0.9	0.089
361	R. L. 8	4 3	14,133	Normal	0.084	0.009	11.0	3.2	0.026
349	H. J. 1	1 4	10,880	Acid	0.084	0.042	50.6	3.3	0.026
360	R. L. 6	4 2	14,093	Normal	0.083	0.011	12.8	3.1	0.027
354	R. L. 7	4 3	14,215	Normal	0.082	0.002	2.6	3.0	0.027
351	F. B. 5	3 9	14,645	Normal	0.082	0.012	8.5	3.0	0.027
355	R. L. 9	4 3	14,668	Normal	0.080	0.029	36.4	3.0	0.027
384	F. B. 6	3 10	14,730	Normal	0.080	-0.007	0.0	2.8	0.029
362	R. L. 10	4 3	14,815	Normal	0.080	0.005	6.8	3.0	0.027
385	F. B. 8	3 10	15,243	Normal	0.079	0.021	26.7	2.7	0.029

365	W. R. 2	4	7	15,412	Acid	0.077	0.025	32.2	4.2	0.018	Corn oil
352	F. B. 7	3	9	15,050	Normal	0.076	-0.001	0.0	2.7	0.028	Fat-free milk, milk butter
353	R. L. 5	4	2	14,115	Normal	0.071	0.031	44.0	2.6	0.025	
350	H. J. 2	1	4	10,880	Normal	0.065	0.012	18.3	2.7	0.024	
325	C. M.	5	0	18,450	Normal	0.064	0.022	33.6	2.0	0.032	
339	E. A. 1	7	0	11,780	Acid	0.061	0.011	18.1	2.7	0.022	Skimmed milk. High proportion carbohydrate in diet
348	M. J. 3	3	4	15,400	Normal	0.055	0.023	42.3	2.1	0.027	High proportion carbohydrate
347	M. J. 2	3	0	15,400	Acid	0.050	0.014	27.3	2.1	0.024	High proportion carbohydrate
337	D. K.	4	0	18,500	Acid	0.048	0.018	38.2	2.5	0.019	
345	E. H.	3	4	14,700	Normal	0.045	0.008	16.7	1.7	0.027	Very low calories per kilogram
336	R. K.	6	0	20,500	Acid	0.044	0.017	39.3	2.3	0.020	Very low calories per kilogram
'310	L. S.	3	2	16,000	Acid	0.043	0.013	30.4	1.9	0.023	Very low calories per kilogram
338	R. N.	5	10	20,200	Acid	0.018	-0.001	0.0	1.2	0.015	No milk, high proportion carbohydrate, much vegetable and fruit

The absorption of calcium oxid per kilo also was much lower than was found with the infants; it ranged from 0 to 0.147 gm. of calcium oxid per kilo, the two highest values being found when the intake was exceptionally high.

The relation of the absorption to the intake is summarized in Table 3. When the intake of calcium oxid exceeded 0.09 gm. per kilo, the absorption in most cases exceeded 0.03 gm. per kilo; the average was 0.055 gm. per kilo. When the intake was 0.09 gm. per kilo or less, the absorption in most cases was less than 0.03 gm.; the average was only 0.015 gm. per kilo. This absorption would hardly supply as much calcium oxid as is normally excreted in the urine by young children. It may, therefore, be inferred that an intake of less than 0.09 gm. of calcium oxid per kilo is insufficient to supply the calcium need of young children taking a mixed diet.

TABLE 3.  
*Relation of Calcium Absorption to Calcium Intake.*

Calcium Oxid Intake, Gm. per Kg	Number of Observations	Absorption 0.09 Gm. or More	Absorption 0.06 to 0.09 Gm.	Absorption 0.03 to 0.06 Gm.	Absorption Less Than 0.03 Gm.
More than 0.09 gm.....	45	7	7	24	7
Less than 0.09 gm.....	34	0	0	3	31
Total.....	79	7	7	27	38

*Relation of Calcium Absorption to Fat Intake.*—With the older children who were taking a mixed diet, there was great variation in the intake of fat per kilo. On the whole, there was no close relation between the fat intake and the calcium absorption. When the fat intake was high, frequently the calcium intake also was high, and consequently there was good absorption of calcium. The intake generally included less calcium in proportion to the fat than in the case of infants who were taking modifications of cow's milk. In but few instances was there more than 0.05 gm. of calcium oxid per gm. fat in the intake, and in many there was less than 0.03 gm. The highest absorption occurred when the fat intake was greater than 3.0 gm. per kilo and when for every gram of fat in the diet there was from 0.03

to 0.05 gm. of calcium oxid, that is, when the calcium intake exceeded 0.09 gm. of calcium oxid per kilo.

*Percentage Absorption of Calcium.*—The percentage of the calcium intake absorbed had a very wide range, varying from 0 to 66.6 per cent. When the calcium intake exceeded 0.09 gm. of calcium oxid per kilo, the percentage absorption averaged 40.4; when the intake was 0.09 gm. or less, the average absorption was only 20.3 per cent. of the intake. The excretion of calcium oxid in the stools of healthy

TABLE 4.  
*Average Absorption of Calcium Oxid According to Weight.*

Weight in Kilograms	Number of Observations	Calcium Oxid Intake, Gm. per Kg	Calcium Oxid Absorbed, Gm. per Kg	Percentage of Calcium Oxid Intake Absorbed
<b>A. When Intake Exceeded 0.09 Gm. per Kg.</b>				
7-9.....	7	0.189	0.068	36.0
9-11.....	9	0.150	0.072	48.0
11-13.....	10	0.128	0.046	36.0
13-15.....	10	0.115	0.044	38.2
15-17.....	9	0.113	0.051	45.2
Under 11.....	16	0.167	0.070	41.9
Over 11.....	29	0.119	0.046	38.7
<b>B. When Intake Was 0.09 Gm. per Kg. or Less</b>				
Under 13.....	5	0.076	0.014	18.5
13-15.....	15	0.081	0.013	16.1
15-17.....	10	0.073	0.020	27.4
Over 17.....	4	0.044	0.014	31.8

children taking a mixed diet did not fall below a certain minimum, whatever the calcium intake. Thus the low intake of calcium oxid did not supply much excess over the amount which was normally excreted in the stools. Hence, the amount available for absorption when the intake was low was much less than the amount which was actually found to be absorbed when the intake was ample. This explains why, with a small intake, the percentage absorption was lower than with a larger intake, since the excretion tends to remain constant at the expense of the absorption. This observation is paralleled by

a similar one in regard to fat, noted in a previous paper,<sup>1</sup> namely, that the percentage absorption of fat was lower when the intake was small than when it was ample.

*Relation of Calcium Absorption to Body Weight.*—The absorption of calcium oxid per kilo was to a certain extent related to the total body weight, as is shown in Table 4. With an adequate intake, that is, exceeding 0.09 gm. of calcium oxid per kilo, the children weighing less than 11 kilos absorbed, on the average, about 0.07 gm. per kilo, while those weighing more than 11 kilos absorbed, on the average, only about 0.05 gm. per kilo. The infants, considered in the preced-

TABLE 5.

*Average Absorption of Calcium Oxid According to Type of Stool.*

Type of Stool	Number of Observations	Calcium Oxid Intake, Gm. per Kg.	Calcium Oxid Absorbed, Gm. per Kg.	Percentage of Calcium Oxid Intake Absorbed
A. When Intake Exceeded 0.09 Gm. per Kg.				
Constipated.....	16	0.149	0.064	43.0
Normal.....	24	0.130	0.048	36.9
Acid.....	5	0.122	0.057	46.7
B. When Intake Was 0.09 Gm. per Kg. or Less				
Normal.....	24	0.080	0.015	18.8
Acid.....	10	0.060	0.017	28.3

ing paper, most of whom weighed less than 7 kilos, had an average absorption of about 0.09 gm. of calcium oxid per kilo. With the children taking a mixed diet whose intake was less than 0.09 gm. per kilo the absorption showed little variation with the weight.

There was no constant relation between the calcium absorption and the age, irrespective of the weight.

*Relation of Calcium Absorption to Type of Stool.*—The relation of the calcium absorption to the type of stool is shown in Table 5. The best average absorption was found when the stools were constipated, but the average intake for this group was the highest. Acid stools were rarely found when the intake was over 0.09 gm. per kilo. In

1. Am. J. Dis. Child. 17: 423 (June) 1919.

the few instances when the stools were acid, the intake being adequate, the average absorption was as good as when the stools were constipated or normal. Also, when the intake of calcium oxid was less than 0.09 gm. per kilo, the absorption was practically the same whether the stools were acid or alkaline, but much lower than when the intake was adequate. That the absorption of calcium oxid was dependent more on the amount of the calcium intake than on the type of stool is shown by comparing the values for the normal groups in Table 5. With high intake, the absorption was about three times as great as with low intake. The influence of the amount of intake of calcium oxid on both the type of stool and the absorption of calcium oxid is further illustrated by three observations on one child, M. J. (Tables 1 and 2). When the intake of calcium oxid was 0.093 gm. per kilo, the stools were constipated and the absorption was 0.058 gm. per kilo; when the intake was 0.056 gm., the stools were normal and the absorption was only 0.023 gm.; again, when the intake was 0.050 gm., the stools were acid and the absorption was only 0.014 gm.

Tables 6 and 7 show for the cases shown in Tables 1 and 2 the calcium excretion in the stools and its relation to the total calcium intake, to the fat intake and to the excretion of total fat and of fat as soap.

*Calcium Excretion and Its Relation to Calcium and Fat Intake.*—Excluding two exceptionally high values, the total daily excretion of calcium oxid ranged from 0.45 to 1.37 gm., averaging 0.87 gm. Nearly two-thirds of the values were below 1.0 gm. The excretion was but little related to the intake of calcium oxid. When the intake was low, the excretion did not fall much below the average, so that it formed a much larger proportion of the intake than when the intake was high.

*There was no evidence that a very large intake of fat was followed by an excessive excretion of calcium.* In fact, no relation was shown between the excretion of calcium and the intake of fat.

*Relation of Calcium Excretion to Excretion of Total Fat and of Fat as Soap.*—There was but little relation between the calcium excretion and the total fat excretion. Table 6, which gives the instances in which the calcium excretion was greatest, shows only a few more

TABLE 6.

*Excretion of Calcium by Healthy Children Taking a Mixed Diet; (1) with Calcium in Stools Exceeding 0.9 Gm.*

No.	Case	Stools	CaO	Fat	CaO in	Fat in	Fat as	CaO Possibly	
			Intake, Gm. Daily	Intake, Gm. Daily	Stools, Gm. Daily	Stools, Gm. Daily	Soap, Gm. Daily	CaO in Stools	CaO In- take
295	M. M.	Normal	1.94	38.8	1.70	1.56	0.53	3.1	2.7
225	C. A. 1	Normal	1.85	35.0	1.58	4.10	2.83	17.9	15.3
296	E. M. 1	Constipated	1.84	38.8	1.37	2.29	0.66	4.8	3.6
330	H. F.	Normal	1.76	38.3	1.37	1.62	0.67	4.9	3.8
234	A. L.	Normal	1.08	21.8	1.27	1.81	0.56	4.4	5.2
384	F. B. 6	Normal	1.17	40.9	1.27	2.09	1.07	8.4	9.2
301	F. B. 2	Acid	1.42	43.4	1.26	3.53	1.06	8.4	7.5
307	H. F. 3	Constipated	1.38	36.5	1.24	2.55	1.31	10.6	9.5
313	E. K.	Normal	1.75	37.8	1.24	3.57	1.51	12.2	8.6
233	F. W. 8	Normal	1.60	31.0	1.21	4.10	2.41	19.9	15.1
276	R. L. 2	Normal	1.36	48.1	1.21	1.82	1.00	8.3	7.3
254	E. M. 6	Normal	1.21	37.7	1.20	2.94	1.55	12.9	12.8
311	R. K. 2	Constipated	1.60	31.0	1.18	4.10	1.68	5.8	4.3
352	F. B. 7	Normal	1.16	41.1	1.18	2.85	1.42	12.0	12.2
366	W. R. 3	Normal	1.76	77.0	1.18	4.66	2.26	19.2	12.9
379	H. F. 4	Normal	1.36	37.3	1.17	2.56	1.03	8.8	7.5
274	A. W. 5	Normal	1.36	52.1	1.17	2.56	1.06	9.1	7.8
287	W. W.	Normal	1.67	50.9	1.17	2.40	0.99	8.5	5.9
383	E. M. 7	Normal	1.22	38.1	1.16	2.37	1.04	9.0	8.5
289	M. C.	Normal	1.83	44.5	1.15	1.70	0.71	6.2	3.9
354	R. L. 7	Normal	1.16	43.2	1.13	3.29	1.36	11.9	11.6
327	R. M.	Acid	1.83	50.5	1.11	4.35	1.23	11.1	6.7
308	H. F. 5	Constipated	1.38	35.2	1.10	1.96	1.20	10.9	8.7
362	R. L. 10	Normal	1.18	44.6	1.10	1.68	0.79	7.2	6.7
367	W. R. 4	Normal	1.76	77.0	1.08	3.87	1.35	12.5	7.7
361	R. L. 8	Normal	1.18	45.4	1.05	1.75	0.79	7.5	6.7
324	T. R.	Constipated	1.76	38.3	1.04	1.49	0.55	5.3	3.1
360	R. L. 6	Normal	1.17	43.2	1.02	1.86	0.58	5.7	5.0
351	F. B. 5	Normal	1.20	44.4	1.02	2.74	1.46	14.3	12.1
290	D. R.	Constipated	1.36	44.8	1.01	1.26	0.38	3.8	2.8
232	J. E.	Normal	1.51	28.0	0.99	1.92	1.10	11.1	7.3
302	F. B. 3	Normal	1.41	42.5	0.99	2.25	0.75	7.6	5.3
299	E. M. 4	Normal	1.22	40.1	0.97	2.53	0.90	9.3	7.4
267	O. W. 1	Normal	1.75	37.8	0.94	1.89	1.08	11.5	6.2
380	H. F. 6	Normal	1.38	35.3	0.93	2.24	0.74	8.0	4.9
264	F. W. 9	Normal	1.21	43.6	0.93	2.92	1.45	15.6	12.0
382	E. M. 5	Normal	1.22	39.2	0.93	1.84	0.66	7.1	5.4
300	F. B. 1	Normal	1.81	34.3	0.90	2.16	1.52	16.9	8.4

TABLE 7.

*Excretion of Calcium by Healthy Children Taking a Mixed Diet; (2) with Calcium in Stools Less Than 0.9 Gm.*

No.	Case	Stools	CaO Intake, Gm. Daily	Fat Intake, Gm. Daily	CaO in Stools, Gm. Daily	Fat in Stools, Gm. Daily	Fat as Soap, Gm. Daily	CaO Possibly Held as Soap, Percentage of	
								CaO in Stools	CaO Intake
328	D. D.	Acid	1.34	41.3	0.88	1.63	0.80	9.1	6.0
270	A. W. 1	Constipated	1.76	38.4	0.88	1.43	0.67	7.6	3.8
385	F. B. 8	Normal	1.20	41.8	0.88	1.42	0.41	4.7	3.4
305	H. F. 1	Constipated	1.69	34.9	0.87	1.68	0.90	10.3	5.3
321	F. S.	Constipated	2.19	61.0	0.87	2.46	1.26	14.5	5.8
317	K. P.	Normal	1.29	34.5	0.84	1.23	0.40	4.8	3.1
297	E. M. 2	Normal	1.18	32.6	0.84	1.95	0.69	8.2	5.8
312	R. K. 3	Normal	1.76	38.3	0.83	0.95	0.39	4.7	2.2
291	R. K.	Normal	1.50	42.5	0.83	2.18	1.21	14.6	8.1
298	E. M. 3	Normal	1.21	34.1	0.83	2.53	0.94	11.3	7.8
373	F. W. 7	Normal	1.21	59.4	0.80	3.01	0.44	5.5	3.6
320	J. O. 2	Constipated	1.76	38.3	0.80	1.20	0.68	8.3	3.9
365	W. R. 2	Acid	1.18	64.5	0.80	2.57	0.97	12.1	8.1
325	C. M.	Normal	1.19	36.8	0.79	1.35	0.33	4.2	2.8
277	R. L. 3	Acid	1.55	52.1	0.76	1.18	0.25	3.3	1.6
355	R. L. 9	Normal	1.18	44.6	0.75	3.34	1.08	14.4	9.6
318	D. L.	Constipated	1.45	33.6	0.73	4.04	2.64	36.2	18.2
369	W. R. 6	Normal	2.18	63.1	0.73	1.50	0.44	6.0	2.0
309	H. F. 7	Constipated	1.17	39.8	0.72	2.71	0.73	10.1	6.2
372	F. W. 6	Normal	1.21	59.4	0.72	3.03	0.66	9.2	5.5
326	E. A. 2	Constipated	1.25	34.0	0.72	4.17	2.35	32.9	18.8
364	O. W. 4	Normal	1.79	47.5	0.69	2.10	0.93	13.5	5.2
271	A. W. 2	Normal	1.36	48.1	0.68	1.21	0.50	7.4	3.7
248	E. D.	Acid	0.86	9.7	0.67	4.42	1.83	27.3	21.3
269	O. W. 5	Constipated	1.76	53.5	0.66	3.20	1.69	25.6	9.6
359	R. L. 4	Acid	1.39	63.6	0.66	3.94	1.44	21.8	10.4
268	O. W. 2	Constipated	1.76	38.5	0.65	2.16	1.40	21.5	8.0
339	E. A. 1	Acid	0.72	32.2	0.59	1.51	0.49	8.3	6.8
272	A. W. 3	Normal	1.36	52.1	0.59	2.20	0.89	15.1	6.5
350	H. J. 2	Normal	0.71	29.1	0.58	1.35	0.40	6.9	5.6
353	R. L. 5	Normal	1.00	40.0	0.56	3.57	1.93	34.5	19.3
347	M. J. 2	Acid	0.77	32.4	0.56	4.44	0.41	7.3	5.3
345	E. H.	Normal	0.66	24.5	0.55	1.06	0.55	10.0	5.3
337	D. K.	Acid	0.89	46.6	0.55	1.31	0.07	1.3	0.8
322	M. J. 1	Constipated	1.43	48.2	0.54	1.04	0.31	5.7	2.2
336	R. K.	Acid	0.89	45.6	0.54	1.62	0.21	3.9	2.4
338	R. N.	Acid	0.36	23.9	0.51	1.43	0.40	7.9	11.1
348	M. J. 3	Normal	0.85	31.3	0.49	2.06	0.40	8.2	4.7
340	L. S.	Acid	0.69	30.5	0.48	1.58	0.52	10.8	7.5
381	H. F. 8	Acid	1.17	43.7	0.46	2.07	1.07	23.2	9.1
349	H. J. 1	Acid	0.91	35.5	0.45	1.10	0.35	7.8	3.9

instances of high fat excretion than does Table 5, which gives the instances in which the calcium excretion was lowest.

In order to bring out the relation between soap excretion and calcium excretion, averages were found for the different types of stools, since the average soap excretion varies according to the type of stool. These averages are given in Table 8.

Table 8 A shows that in the constipated stools there was a greater soap excretion and a smaller calcium oxid excretion than in the normal stools, although when the stools were constipated, the intake was much greater. Table 8 B, which compares with the group of con-

TABLE 8.  
*Average Excretion of Calcium According to Type of Stool.*

Stools	Number of Observa- tions	Intake of CaO, Gm. Daily	CaO in Stools, Gm. Daily	Fat in Stools, Gm. Daily	Fat as Soap, Gm. Daily
<b>A:</b>					
Constipated.....	16	1.60	0.90	2.36	1.15
Normal.....	48	1.38	0.98	2.33	1.00
Acid.....	15	1.06	0.69	2.45	0.74
<b>B:</b>					
Constipated.....	16	1.60	0.90	2.36	1.15
Normal—high calcium oxid intake.....	24	1.63	1.05	2.35	1.10
<b>C:</b>					
Normal—high calcium oxid in stools.....	24	1.46	1.19	2.59	1.20
Normal—low calcium oxid in stools.....	24	1.31	0.77	2.07	0.80

stipated stools a group of normal stools when the average intake was similar, brings out more clearly the contrast between these two types of stools in calcium and soap excretion.

However, excluding the constipated stools, there was a general correspondence between the variations in calcium excretion and in soap excretion. The average for the acid stools showed a much lower soap excretion, with a much lower calcium excretion, than did the normal stools, but the intake for this group was also much lower than that for the normal group. As was previously noted in the discussion of calcium absorption, the calcium intake apparently influences to a considerable extent the reaction of the stools, a low calcium intake frequently resulting in acid stools.

The relation between calcium and soap excretion is independent of the calcium intake, as is brought out in Table 8 C, in which averages are given for two groups of normal stools arranged according to the calcium excretion. With similar intake the average soap excretion in these two groups of normal stools was exactly proportional to the calcium excretion.

Both with infants fed on milk modifications and with older children taking a mixed diet there was, as has been stated, less calcium excreted in proportion to the amount of soap when the stools were constipated than when the stools were normal. This indicates that there was on the average proportionally less calcium phosphate in the constipated than in the normal stools.

*The calcium lost as soap was in most cases an insignificant part of the calcium intake.* In only fourteen of the seventy-nine instances in Tables 6 and 7 did it exceed 10 per cent. In the majority of the cases it was less than 10 per cent. of the total calcium excretion, and in only eight instances did it exceed 20 per cent.

#### *Effect of Vegetable Fats on Calcium Metabolism.*

In our study of fat metabolism, one group of children was given corn oil in place of milk fat, and another group nut butter in place of milk fat. The effect of these vegetable fats on calcium metabolism is shown by the averages in Table 9.

In the nut butter series the children received in other respects the same diet as during the periods in which the fat given was milk fat. Hence, the findings for these two groups in Table 9 are exactly comparable. There were no control observations to be compared with the observations on corn oil. Accordingly, an average has been found for the cases given in Tables 1, 2, 6 and 7, in which the fat in the diet was mainly milk fat and the intake of calcium was comparable with that of the children taking corn oil.

Table 9 shows that the calcium absorption and the excretion were practically the same whether the fat in the diet was milk fat or vegetable fat. *The only difference in the calcium metabolism was in the proportion of the calcium excreted in the form of soap.* With the children taking nut butter, the soap excretion was markedly lower than

with milk butter and, therefore, a smaller proportion of the calcium excretion could have been bound as soap. With the children taking corn oil the soap excretion was much greater than that of the group of children with similar calcium intake who were taking mainly milk fat. The children taking corn oil received an unusually high intake of fat, so that in respect to fat excretion the two groups are not comparable.

TABLE 9.

*Average Absorption and Excretion of Calcium with Milk Fat and with Vegetable Fat.*

Kind of Fat in Diet	Number of Observations	CaO Intake, Gm. per Kg.	CaO Absorbed, Gm. per Kg.	Total CaO Intake, Gm. Daily	Total CaO in Stools, Gm. Daily	Fat as Soap in Stools, Gm. Daily
Milk butter.....	9	0.100	0.016	1.21	1.02	1.36
Nut butter.....	9	0.100	0.015	1.23	1.06	0.79
Mainly milk fat.....	35	0.119	0.047	1.52	0.95	0.97
Corn oil.....	13	0.122	0.052	1.56	0.89	1.40

### Rachitic Children.

All the children classed here as rachitic showed, at the time of coming under observation, marked rachitic symptoms, such as inability to stand or walk, distended abdomen and typical bone changes. With most of the children observations were made before treatment was begun, as well as during the period of recovery. Table 10 gives the absorption of calcium oxid per kilo and its relation to the calcium and the fat intake.

*Calcium Absorption.*—The intake of calcium oxid per kilo was high, in most cases higher than that of the majority of the normal children and in no case was it less than 0.09 gm., which, with normal children, appears to be an adequate intake. In the five observations made before treatment was begun, noted in Table 8 as preliminary observations, the absorption of calcium oxid was low, mostly below the normal average. The highest absorption occurred when the intake was very high. As the condition of the children improved, the calcium absorption increased to well above the normal average, as is shown by the later observations on all the children. With two of

TABLE 10.  
*Absorption of Calcium by Rachitic Children Taking Mixed Diet.*

No.	Case	Age Yr. Mo.	Weight in Gm.	Stools	Intake of CaO, Gm. per Kg.	CaO Absorbed, Gm. per Kg.	Percentage of CaO ab- sorbed	Grams CaO Intake per Gm. Fat Intake	Remarks
235	L. H. 2	1 6	8,270	Acid	0.288	0.160	55.4	5.5	0.053 Protein milk
265	I. H. 1	2 6	6,560	Normal	0.268	0.228	84.7	8.9	0.030 Cod liver oil, high calories per kilogram
266	I. H. 2	2 8	7,045	Normal	0.248	0.189	76.4	6.7	0.037 Cod liver oil, high calories per kilogram
316	L. R. 2	1 11	8,550	Normal	0.244	0.165	67.5	6.1	0.040 Cod liver oil just discontinued
262*	F. W. 2	3 2	9,207	Normal	0.238	0.059	24.8	3.7	0.065 Butter in place of cod liver oil of previous period.
315	L. R. 1	1 10	8,205	Normal	0.223	0.127	56.8	4.9	0.046 Cod liver oil just discontinued
257	R. M. 3	2 8	9,916	Normal	0.216	0.113	52.1	6.2	0.035 Low carbohydrate
256	R. M. 2	2 7	8,570	Constipated	0.199	0.090	45.2	6.5	0.031 Cod liver oil. Low carbohydrate
261*	F. W. 1	3 1	8,900	Constipated	0.182	0.021	11.8	2.7	0.067 Olive oil in place of butter of previous period
263	F. W. 3	3 4	10,110	Normal	0.179	0.097	54.1	5.6	0.032 Cod liver oil
304*	R. A. 1	4 0	7,917	Acid	0.176	0.037	26.4	5.3	0.033 Extra butter
253*	R. M. 1	2 5	8,017	Acid	0.163	0.026	16.1	2.4	0.067 Protein milk and whole milk. Very low calories per kilogram
258	R. M. 4	2 10	10,825	Constipated	0.151	0.068	44.7	4.3	0.035 Extra butter
356	R. A. 2	4 3	9,515	Acid	0.150	0.084	56.3	5.4	0.028 Extra butter
357	R. A. 3	4 4	10,303	Acid	0.138	0.079	57.0	5.6	0.024 Extra butter
333*	L. H. 1	1 5	8,060	Acid	0.126	0.051	40.6	4.2	0.030 Fat-free milk with corn oil
370	F. W. 4	3 6	10,700	Normal	0.113	0.080	71.1	5.6	0.020 Fat-free milk with corn oil
371	F. W. 5	3 6	10,750	Normal	0.113	0.044	38.8	5.5	0.020 Fat-free milk with corn oil

\* Preliminary observation.

the children later observations (371,258) made after recovery was well established, showed a subsequent decrease in absorption. These observations confirm the conclusions of other investigators that *the absorption of calcium is low when active rickets is present, increased above the normal during recovery and falls as the need for calcium ceases to be greater than normal.* It must be remembered that by calcium absorption is here meant the difference between the calcium intake and the amount lost in the stools.

The treatment in our cases consisted in a change of the diet to include more calcium and fat by increasing the milk and giving cod liver oil or milk butter, thus furnishing an extra amount of "fat-soluble A." In the latest observations on F. W. and R. M., made after recovery was well established, vegetable fat replaced butter and cod liver oil.

In the preliminary observations, the percentage of the calcium intake absorbed was with one exception much lower than normal, but during the period of improvement, the percentage absorbed was much higher than with normal children.

The intake of fat per kilo was very high, except in some of the preliminary observations, but, in general, the ratio of the calcium to the fat of the intake was similar to that of the normal children. This resulted from the increase of both fat and calcium in the diet as a therapeutic measure.

Table 11 gives for the rachitic children the calcium excretion in the stools in relation to the calcium and fat intake and to the excretion of total fat and of fat as soap.

*Excretion of Calcium.*—The range in calcium excretion was about the same as in the case of the normal children. There was no constant relation between the calcium excretion and the intake of either calcium or fat. On the whole, the calcium excretion was not related to the fat excretion. As with normal children, the excretion of soap was sometimes parallel with that of calcium and sometimes not. The lowest values for soap excretion were found when the calcium excretion was lowest, but the highest calcium excretion was not accompanied by the highest soap. In most cases the stools of these rachitic children contained more soap but not more calcium than those of normal children, so that the calcium lost as soap was a distinctly

higher proportion of both the calcium excretion and the calcium intake than was found with normal children.

*Effect of Treatment.*—There was great difference in the calcium metabolism between the period of active rickets and that of recovery. Accordingly, averages are given in Table 12 representing the preliminary and the later observations. Observations 258, 370 and 371

TABLE 11.  
*Excretion of Calcium by Rachitic Children Taking a Mixed Diet.*

No.	Case	Stools	CaO	Fat	CaO in	Fat in	Fat as	CaO Possibly	
			Intake, Gm. Daily	Intake, Gm. Daily	Stools, Gm. Daily	Stools, Gm. Daily	Soap, Gm. Daily	Held as Soap, Percentage of	
								CaO in Stools	
								CaO Intake	
262*	F. W. 2	Normal	2.18	33.8	1.64	2.49	1.79	10.9	8.2
235	L. H. 2	Acid	2.38	45.1	1.32	5.71	2.17	20.5	9.1
261*	F. W. 1	Constipated	1.62	24.2	1.43	2.97	1.82	12.8	11.2
304*	R. A. 1	Acid	1.39	42.1	1.10	3.61	2.00	18.2	14.4
253*	R. M. 1	Acid	1.31	19.6	1.10	4.24	1.84	16.7	14.0
257	R. M. 3	Normal	2.15	61.0	1.03	6.42	2.87	27.8	13.3
256	R. M. 2	Constipated	1.70	55.7	0.93	8.59	4.19	45.1	24.7
258	R. M. 4	Constipated	1.63	47.2	0.90	7.34	3.34	37.1	20.5
263	F. W. 3	Normal	1.81	56.7	0.83	3.44	2.05	24.7	11.3
315	L. R. 1	Normal	1.83	39.8	0.79	1.96	1.23	15.6	6.7
371	F. W. 5	Normal	1.21	59.4	0.74	5.30	3.00	40.6	24.8
316	L. R. 2	Normal	2.09	52.5	0.68	3.56	2.29	33.7	11.0
356	R. A. 2	Acid	1.42	51.5	0.62	2.17	0.97	15.7	6.8
357	R. A. 3	Acid	1.42	58.1	0.61	2.33	1.26	20.7	8.9
333*	L. H. 1	Acid	1.01	33.5	0.60	2.62	0.51	8.5	5.0
266	I. H. 2	Normal	1.74	46.9	0.41	1.58	0.64	15.6	3.7
370	F. W. 4	Normal	1.21	59.4	0.35	4.01	1.39	39.7	11.5
265	I. H. 1	Normal	1.76	58.3	0.27	1.49	0.94	34.8	5.3

\* Preliminary observation.

are not included in the average, because they represent periods after recovery was well established and in which the food included much vegetable fat.

The average calcium excretion was much higher during active rickets than the normal average and during recovery somewhat lower than the normal average. The average intake of calcium oxid' was somewhat higher in the period of recovery, making the percentage

retention much greater. Although during recovery the fat intake was nearly doubled, the excretion of total fat and of fat as soap was but slightly increased and the percentage absorption of fat as well as of calcium was much improved.

With four of the children, the extra fat was provided by the addition of cod liver oil to the diet. With one child, additional fat was given as butter, with as good result as when cod liver oil was used. Another child showed an improved absorption of calcium on a special "protein milk" in which calcium phosphate and fat were both much increased.

TABLE 12.

*Excretion of Calcium by Children with Active Rickets and Those Recovering from Rickets.*

Condition of Children	No. of Observations	CaO Intake, Gm. Daily	Fat Intake, Gm. Daily	CaO in Stools, Gm. Daily	Fat in Stools, Gm. Daily	Fat as Soap, Gm. Daily	CaO as Soap, Percentage of		Percentage of CaO Intake Absorbed	Percentage of Fat Intake Absorbed
							CaO in Stools	CaO Intake		
With active rickets.....	5	1.50	30.6	1.17	3.19	1.59	13.6	10.6	22.0	89.6
Recovering from rickets...	10	1.83	57.6	0.75	3.73	1.86	24.8	10.2	59.0	93.6

#### *Children Suffering from Chronic Intestinal Indigestion.*

Table 13 presents the findings for the absorption of calcium per kilo and its relation to the calcium and the fat intake for children suffering from chronic intestinal indigestion.

*Calcium Absorption.*—The intake of calcium oxid per kilo was in general lower than that of the normal children, and, except in a few instances, the absorption was extremely low. There were only four values for absorption exceeding 0.04 gm. per kilo, and these occurred when the fat intake exceeded 4.0 gm. per kilo. Only once, with as high an intake of fat, was the absorption less than 0.04 gm. per kilo. A high intake of calcium did not regularly result in high absorption, although in no case was there good absorption when the intake of calcium oxid was less than 0.10 gm. per kilo. The percentage of the calcium intake absorbed was, as a rule, very low.

As has been previously observed, *the absorption of calcium by in-*

TABLE 13.  
*Absorption of Calcium by Children Suffering from Chronic Intestinal Indigestion.*

No.	Case	Age Yr. Mo.	Weight in Gm.	Stools	CaO Absorbed Gm. per Kg.	Percentage of CaO Intake Absorbed	Grams CaO Intake Gm. per Kg.	CaO Intake Gm. per Fat Intake	Remarks
332	N. C. 1	6 0	8,655	Alkaline	0.242	-0.080	0.0	2.7	0.087
331	N. C. 2	6 4	8,705	Alkaline	0.241	0.003	1.4	4.3	0.056
243	H. F. 1	2 10	9,830	Acid	0.202	0.015	7.5	1.7	0.116
249	S. M.	2 10	7,685	Acid	0.191	0.113	59.2	4.8	0.040
236	G. R. 1.	5 0	12,727	Alkaline	0.158	0.032	25.6	1.0	0.158
294	M. H.	2 1	7,150	Acid	0.150	0.066	43.8	4.3	0.035
334	H. L. 1	8 0	14,392	Acid	0.144	0.031	21.7	2.3	0.062
237	G. R. 2	5 8	14,716	Alkaline	0.140	-0.026	0.0	2.5	0.058
240	F. G. 2	5 5	13,060	Acid	0.133	0.051	37.7	5.7	0.023
242	F. G. 4	6 5	17,140	Alkaline	0.114	0.007	6.2	1.9	0.059
341	A. E.	2 9	5,165	Alkaline	0.110	0.027	24.7	3.0	0.037
244	H. F. 2	3 3	10,000	Acid	0.106	0.047	44.3	4.0	0.027
241	F. G. 3	6 0	17,000	Alkaline	0.102	0.014	13.9	3.5	0.029
255	D. R.	3 0	10,960	Acid	0.089	0.032	36.1	2.2	0.041
238	G. R. 3	7 8	23,600	Acid	0.083	0.019	22.4	2.1	0.040
323	E. R. 2	9 4	16,590	Alkaline	0.083	0.014	17.3	2.9	0.028
239	G. R. 4	8 0	22,850	Alkaline	0.081	0.031	37.8	2.4	0.034
278	W. R. 1	4 6	14,659	Acid	0.071	0.004	5.8	2.4	0.030
343	H. L. 2	8 0	14,150	Mixed with urine	0.042	-0.014	0.0	2.1	0.020
335	E. R. 1	8 5	12,550	Alkaline	0.041	-0.015	0.0	2.3	0.018
342	F. G. 1	5 0	10,508	Acid	0.013	0.004	28.6	2.0	0.007

fants and young children was not good unless the calcium intake bore a suitable ratio to the fat intake. With normal children, taking a mixed diet, apparently the food should contain from 0.03 to 0.05 gm. of calcium oxid for every gram of fat, and, at the same time, the intake of both calcium and fat should be sufficient. It is seen in Table 13 that in the instances in which the calcium intake was high and the absorp-

TABLE 14.

*Excretion of Calcium by Children Suffering from Chronic Intestinal Indigestion.*

No.	Case	Stools	CaO Intake, Gm. Daily	Fat Intake, Gm. Daily	CaO in Stools, Gm. Daily	Fat in Stools, Gm. Daily	Fat as Soap, Gm. Daily	CaO Possibly Held as Soap, Percentage of	
								CaO in Stools	CaO Intake
332	N. C. 1	Alkaline	2.09	23.5	2.78	3.98	1.58	5.7	7.6
237	G. R. 2	Alkaline	2.06	35.8	2.45	11.20	7.90	32.2	38.3
331	N. C. 2	Alkaline	2.10	37.6	2.07	5.99	1.83	8.7	8.7
243	H. F. 1	Acid	1.99	17.1	1.84	12.50	3.11	16.9	15.6
242	F. G. 4	Alkaline	1.95	33.0	1.83	6.19	3.50	19.1	17.9
334	H. L. 1	Acid	2.07	33.3	1.62	11.34	3.21	19.8	15.5
238	G. R. 3	Acid	1.96	49.0	1.52	7.49	4.05	26.6	20.6
241	F. G. 3	Alkaline	1.73	58.9	1.49	9.54	5.06	33.9	29.2
239	G. R. 4	Alkaline	1.85	55.1	1.15	5.11	1.92	16.7	10.5
323	E. R. 2	Alkaline	1.33	48.4	1.10	14.64	4.72	42.9	35.4
240	F. G. 2	Acid	1.73	74.2	1.06	18.67	5.66	53.3	32.7
278	W. R. 1	Acid	1.04	35.2	0.98	4.34	1.84	18.8	17.7
343	H. L. 2	Mixed with urine	0.59	29.9	0.79	8.95			
335	E. R. 1	Alkaline	0.51	29.2	0.70	11.20	4.72	67.2	92.4
255	D. R.	Acid	0.97	23.8	0.62	4.68	2.32	37.4	23.9
294	M. H.	Acid	1.07	30.5	0.60	4.79	2.05	34.2	19.2
249	S. M.	Acid	1.47	36.7	0.60	7.30	2.67	44.5	18.2
244	H. F. 2	Acid	1.06	39.8	0.59	3.54	2.07	23.4	18.6
341	A. E	Alkaline	0.57	15.5	0.43	1.95	1.37	31.9	24.0
342	F. G. 1	Acid	0.14	21.0	0.10	4.68	1.25	125.0	89.3

tion poor, the ratio of calcium to fat in the intake was abnormally high. In other words, there was not enough fat in the food for the amount of calcium. In the other instances of poor absorption of calcium, the intake of both fat and calcium was too low.

Table 14 gives for these children the calcium excretion in the stools and its relation to the calcium and the fat intake and to the excretion of total fat and of fat as soap.

*Calcium Excretion.*—In general, the calcium excretion was much higher than with normal children. When the excretion of calcium oxid was low, the intake was usually very low. There was no constant relation between the intake of fat and the excretion of calcium in the stools. The excretion of fat was in most cases extremely high, only once being less than 3.5 gm. The variation in the excretion of total fat and of fat as soap was entirely unrelated to the calcium excretion. Since the excretion of soap was large, the calcium lost as soap formed a large percentage of both the calcium excretion and the calcium intake.

As the condition of chronic intestinal indigestion is characterized by the occurrence of very large stools containing a high proportion of fat, there is a temptation to withhold fat from the diet of these children. Our findings indicate that to insure a sufficient absorption of calcium, the intake of calcium must be ample and that it must be accompanied by a suitable proportion of fat. For this reason, it appears to be better to give larger amounts of fat with calcium in due proportion to children suffering from chronic intestinal indigestion, even though the loss of fat in the stools is abnormally great. As was shown previously, in our discussion of fat metabolism, these children retained a much larger amount of fat when their intake of fat was greatly increased, although their excretion of fat was at the same time increased.<sup>2</sup>

#### *Effect on Calcium Metabolism of Variations in Calcium and Fat Intake.*

Table 15 A presents a series of observations on one child, B. W., who first came under observation suffering from osteogenesis imperfecta and malnutrition. The first observation showed with a very high calcium intake an unusually high absorption of calcium. At the time of the second observation, with about the same calcium intake, the absorption had returned to normal. The child was then given 1.6 gm. of calcium acetate daily, equivalent to 0.51 gm. calcium oxid. He received this for twelve days, including the period of observation. This salt had a bad effect on the stools, which became

TABLE 15.  
*Effect of Variation in Calcium and Fat Intake.*

283	2	0	10,888	Constipated	0.174	0.052	30.0	3.9	0.045	1.90	42.1	1.33	3.36	1.86	14.0	9.8	0.020	Proportion of fat increased.
					Normal	0.174	0.000	0.0	4.3	0.041	2.01	49.7	2.00	6.44	2.06	10.3	10.3	No cod liver oil
374	2	1	11,531	Normal	0.174	0.068	39.3	4.3	0.040	2.01	49.8	1.22	7.01	2.42	19.8	12.0	0.047	Fat-free milk, corn oil
375	2	1	11,556	Normal	0.244	0.139	56.8	3.8	0.064	2.87	44.5	1.24	4.65	1.57	12.7	5.5	....	Chalk mixture (1.0 gm. calcium oxid) daily.
377	2	2	11,735	Normal	0.243	0.166	68.3	3.8	0.064	2.87	44.5	0.91	3.30	1.35	14.8	4.7	....	Fat-free milk, corn oil
378	2	3	11,777	Normal	0.243	0.166	68.3	3.8	0.064	2.87	44.5	0.91	3.30	1.35	14.8	4.7	....	Chalk mixture (1.0 gm. calcium oxid) daily.
																		Fat-free milk, corn oil
<b>B. Observations on W. R.</b>																		
367	4	7	15,735	Normal	0.111	0.043	38.6	4.9	0.023	1.76	77.0	1.08	3.87	1.35	12.5	7.7	....	Corn oil.
368	4	8	15,516	Normal	0.276	0.149	54.1	3.9	0.073	4.29	60.1	1.97	4.74	2.58	13.1	6.0	....	Same diet, less corn oil.
																		Chalk mixture (2.0 gm. calcium oxid) daily

watery, acid and showed evidences of undigested food. The absorption of calcium oxid was unchanged; the additional calcium oxid in the intake was excreted in the stools, there being no increase in the calcium excreted in the urine. The calcium acetate was discontinued, and after the stools had become normal, there was given 2.7 gm. of tricalcium phosphate daily, equivalent to 1.46 gm. calcium oxid. He received this for eight days, including the period of observation. This salt also affected the stools unfavorably, and as in the case of calcium acetate, the excess calcium oxid was all excreted in the stools.

A few months later this child was studied again. He was then considered normal as to digestion. The first observation showed excellent absorption of calcium oxid, rather above the normal average. The fat in the food was then reduced to about one half, and the loss in fat calories was made up by increasing the carbohydrates and the protein. This made the ratio of calcium oxid to fat in the intake abnormal. The calcium absorption was much reduced, the loss of calcium in the stools becoming much greater, although the excretion of total fat and of fat as soap was less than in the preceding period. The total food was then reduced, involving a considerable reduction in calcium intake. The absorption of calcium became very low and the excretion in the stools, although less than in the preceding period, was still abnormally high. The food was then increased to give an ample and well balanced intake of fat and calcium. The absorption of calcium rose to about the normal average for children taking a mixed diet. The excretion of calcium oxid in the stools was diminished, although still higher than the normal average. The fat in the diet was then supplied entirely in the form of corn oil, and an ample intake of both calcium and fat was provided. The first observation with this diet was made only forty-eight hours after the change. There was a negative balance of calcium and a large excretion of fat in the stools. The next observation, made a few days later, showed normal calcium absorption but no decrease in fat excretion. Later, one ounce of chalk mixture (calcium carbonate), equivalent to 1.00 gm. of calcium oxid, was given daily for six days, including the period of observation. The absorption of calcium was markedly increased, becoming much higher than the normal average; also the fat excretion was diminished. A second observation was made one week later, the

chalk mixture meanwhile having been continued. The absorption of both calcium and fat was further improved. Unfortunately, urinary findings were not obtained, so that it is not known whether the extra calcium absorbed was to any extent excreted in the urine.

Table 15 B shows the effect of the administration of chalk mixture with another child, W. R. This child was given 2 ounces of chalk mixture daily, equivalent to 2.0 gm. of calcium oxid for seven days, including the period of observation. As with B. W., the calcium absorption became higher than the normal average, although in this case the excretion also was appreciably increased. More than one-half of the added calcium oxid was absorbed, that is, more than was absorbed in the observation on B. W.

To summarize, the points brought out in Table 15 are as follows: High absorption of calcium oxid was found with high intake of calcium oxid; calcium absorption was not increased by administration of calcium acetate or of calcium phosphate; calcium absorption was lowered by reduction of fat intake; calcium absorption was further lowered by further reduction in fat intake and reduction in calcium intake; calcium absorption was raised by increase in fat intake to restore suitable ratio of calcium to fat; calcium absorption was only temporarily reduced by substitution of corn oil for milk in the diet; calcium absorption was greatly increased by the administration of calcium carbonate in chalk mixture.

*Proportion of Calcium and of Soap in Stools of Children  
Taking a Mixed Diet.*

Table 16 gives the average proportions of calcium and of soap in stools of various types from children taking a mixed diet.

The calcium percentage of total solids of the stools of children taking a mixed diet was considerably lower than that of the stools similar in water content of infants taking modifications of cow's milk. This is explainable by the presence of food waste, such as cellulose, in the stools of children taking a mixed diet. The constipated stools had a somewhat higher percentage of calcium than the average of all the normal stools. This was related to the calcium intake, as was shown by dividing the normal stools into two groups. The first of

these corresponded to an average intake similar to that for the group of constipated stools, and showed an average value for calcium percentage of total solids even higher than that of the constipated stools. The acid stools contained a smaller proportion of calcium oxid than did the normal. Acid stools occurred more frequently when the intake of calcium was low, but difference in intake did not entirely account for the smaller proportion of calcium. This is shown by com-

TABLE 16.

*Average Proportions of Calcium and of Soap in Stools of Children Taking a Mixed Diet.*

Condition of Children	Stools	Number of Observations	CaO Intake, Gm. Daily	CaO Percentage of Total Solids	CaO Percentage of Total Solids	Fat as Soap, Percentage of Total Solids
Normal.....	Constipated	23	1.62	8.3	41.8	10.1
Normal.....	Normal	45	1.38	7.7	39.1	7.9
Normal—high calcium oxid intake.....	Normal	21	1.66	9.0	42.4	9.2
Normal—low calcium oxid intake.....	Normal	24	1.13	6.3	36.3	6.5
Normal.....	Acid	18	1.10	5.1	33.0	5.9*
Normal—high fat diet, much corn oil.....	Normal	13	1.61	6.0	38.1	9.4
Normal—high fat diet, much corn oil.....	Acid	4	1.48	4.9	31.7	6.7*
Rachitic.....	Normal and constipated	10	1.85	7.7	35.9	17.4
Rachitic.....	Acid	6	1.49	6.1	39.3	10.4*
With chronic intestinal indigestion.....	Alkaline	10	1.62	8.6	38.6	18.2
With chronic intestinal indigestion.....	Acid	9	1.48	4.6	34.8	14.0*
Normal—no milk in diet.....	Acid	1	0.36	1.5	15.4	1.2*
With chronic intestinal indigestion—no milk in diet.....	Acid	1	0.14	0.6	3.7	7.9*

\* Value probably too high since stools were acid.

paring the average for the group of normal stools corresponding to a low intake with the average for the acid stools. The calcium percentage of total solids was somewhat lower in the acid stools than in the normal stools corresponding to the same intake. When the intake included large amounts of corn oil the calcium percentage of total solids of the stools was appreciably lower than when milk fat was taken. The total solids of these stools were increased by the large excretion of fat, while the calcium was not increased in proportion.

The normal stools of rachitic children contained a lower proportion of calcium than did those of normal children. With these children also the total solids of the stools were increased by high fat excretion. The acid stools of rachitic children showed a rather high percentage of calcium than the acid stools of normal children, but the intake of calcium was higher with the rachitic children.

The stools of children suffering from chronic intestinal indigestion had a lower percentage of calcium than those of normal children when the intake was similar. The lower percentage of calcium in the stools of these children, also, was due to a much increased proportion of fat.

The stools of two children who had no milk in the food and consequently a very small calcium intake showed a calcium percentage of solids in the stools far below the usual.

With mixed diet the calcium formed a smaller percentage of the total salts than with milk modifications, and the variations corresponded, in general, with the variations in calcium percentage of total solids.

With mixed diet the average soap percentage of total solids was always low and varied with the calcium percentage of total solids. With children taking large amounts of corn oil the soap percentage of total solids was proportionally higher than the calcium percentage. With the abnormal children the soap percentage was markedly higher than with normal children having the same type of stool, while the calcium percentage showed less difference from the normal.

With mixed diet the variations in calcium percentage of total solids of the stools were not related to the water content, since the stools varied little in this respect. Normally, the two factors which mainly affected the calcium percentage of total solids were the calcium intake and the reaction of the stools.

The following summary gives answers based on our observations to the questions on calcium metabolism stated in the preceding paper.

#### SUMMARY.

1. *Normal Absorption and Excretion of Calcium.*—With children taking a mixed diet, the absorption of calcium per kilo was lower

than that of infants taking modifications of cow's milk, averaging, when the intake was adequate, 0.055 gm. of calcium oxid per kilo.

The average daily excretion of calcium oxid in the stools of children taking a mixed diet was 0.87 gm.

*2. Calcium Absorption and Excretion in Relation to Calcium and Fat Intake.*—With children taking a mixed diet, the intake of calcium oxid per kilo was lower than that of the infants, the average found for seventy-nine cases being 0.108 gm. per kilo. The absorption of calcium oxid when the intake of calcium oxid was more than 0.09 gm. per kilo in nearly every case exceeded 0.03 gm. per kilo, with an average of 0.055 gm. When the intake was only 0.09 gm. per kilo or less, the absorption rarely exceeded 0.03 gm. per kilo, and in several instances there was negative balance; the average was only 0.015 gm.

The percentage of the calcium intake absorbed when the intake exceeded 0.09 gm. per kilo averaged 40.4; when the intake was 0.09 gm. or less, the absorption averaged only 20.3 per cent.

It may, therefore, be inferred that an intake of at least 0.09 gm. of calcium oxid per kilo is necessary to insure a good absorption by children taking a mixed diet.

The best absorption of calcium oxid occurred when the intake of fat exceeded 3.0 gm. per kilo, and when, at the same time, for every gram of fat there was in the diet from 0.03 to 0.05 gm. of calcium oxid. This is a somewhat lower proportion of calcium oxid to fat than was needed to insure good absorption of calcium oxid by infants taking modifications of cow's milk.

When calcium in the form of chalk mixture (calcium carbonate) was added to the diet, there was a greatly increased absorption of calcium. When calcium was added as calcium acetate or as calcium phosphate the absorption was not increased.

The excretion of calcium was not so closely related to the intake of calcium as in the case of infants taking modifications of cow's milk, and was not at all related to the fat intake.

*3. Effect of Very Small Calcium Intake.*—A very small intake of calcium resulted in either an absorption not much greater than the amount normally excreted in the urine or in a negative balance of calcium.

4. *Relation of Age and Weight to Calcium Absorption.*—The average absorption of calcium oxid per kilo was somewhat lower with the larger children, but the age, irrespective of the weight, had no constant relation to the calcium absorption.

5. *Relation Between Excretion of Calcium and Excretion of Total Fat and of Fat as Soap.*—The excretion of calcium in the stools was not at all related to the excretion of total fat, but bore some relation to the excretion of fat as soap. However, in the constipated stools, which contained the most soap, the calcium excretion was not so great as in the normal stools when the intake of calcium oxid was the same.

6. *Loss of Calcium in Soapy Stools.*—The calcium lost as soap in the stools of normal children taking a mixed diet was, in most cases, an insignificant part of the calcium intake.

7. *Calcium Percentage of Total Solids of the Stools.*—The calcium formed a smaller proportion of the total solids of the stools of children taking a mixed diet than of the stools of infants taking modifications of cow's milk. The calcium percentage of total solids was lower in acid than in normal or constipated stools. The two factors chiefly affecting the percentage of calcium in the stools of children taking a mixed diet were the amount of calcium intake and the reaction of the stools. The *soap* percentage of total solids followed on the average the variation in the *calcium* percentage of total solids.

8. *Calcium Absorption and Excretion in (a) Chronic Intestinal Indigestion; (b) Active Rickets; (c) Recovery from Rickets.*—The *absorption* of calcium by children with chronic intestinal indigestion was extremely low. In the only instances in which the absorption was near the normal both calcium and fat intake were high. The *excretion* of calcium in the stools was very high, except when the intake was unusually low. The excretion of total fat and of fat as soap was very high, but was not paralleled by the calcium excretion.

The calcium *absorption* of children with active rickets was lower than that of normal children, even though the calcium intake was ample. The calcium *excretion* in the stools was somewhat higher than the average excretion in the stools of normal children.

During recovery from rickets, the *absorption* of calcium was higher than the average for normal children. This improvement accom-

panied the taking of cod liver oil or additional butter with a diet containing an ample amount of calcium. The calcium *excretion* in the stools of children recovering from rickets was lower than that in the stools of normal children.

*9. Effect on Calcium Metabolism of (a) Cod Liver Oil; (b) Vegetable Fats.*—Cod liver oil increased the absorption of calcium, except in cases in which the intake of calcium or of fat was very low.

The substitution of vegetable fats for milk fat did not affect the calcium metabolism of children taking a mixed diet.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, April, 1920, Vol. xli, No. 4,  
pp. 567-585.]

## STUDIES OF ACIDOSIS.

### XVI. THE TITRATION OF ORGANIC ACIDS IN URINE.

By DONALD D. VAN SLYKE AND W. W. PALMER.

(*From the Hospital of The Rockefeller Institute for Medical Research, and The Chemical Division of the Department of Medicine, Johns Hopkins Medical School, Baltimore.*)

(Received for publication, February 24, 1920.)

#### *Description of Method.*

100 cc. of urine, roughly measured, are thoroughly mixed with 2 gm. of finely powdered calcium hydroxide, allowed to stand about 15 minutes with occasional stirring, and then passed through a dry folded filter. This treatment removes carbonates and phosphates. To 25 cc. of the filtrate in a 125 to 150 cc. test-tube of clear glass<sup>1</sup> one adds 0.5 cc. of 1 per cent phenolphthalein solution, and 0.2 N hydrochloric acid from a burette (amount need not be measured) until the pink color just disappears (pH = approximately 8). 5 cc. of 0.02 per cent tropeolin OO solution are then added. As the indicator solution is added it is thoroughly mixed with the urine by shaking the tube; if this precaution is omitted some of the tropeolin OO may be precipitated. Finally 0.2 N hydrochloric acid is added from the burette until the red color equals that of a standard solution containing 0.6 cc. of 0.2 N HCl, 5 cc. of tropeolin OO solution, and water to a total volume of 60 cc. When the end-point is approached, sufficient water is added to the titrated solution to make its volume equal to that of the 60 cc. standard solution used in a similar tube as a color control.

<sup>1</sup> We use the tubes of Pyrex glass made by the manufacturers for urea determinations by the Van Slyke and Cullen technique. The tubes are 30 mm. inner diameter, 200 mm. long, and uniform in size.

In comparing the color of the titrated solution with that in the standard, it is convenient during the titration to hold the two tubes side by side between the thumb and fingers, the tube containing the urine being the one held nearer to the tips of the fingers where it can be easily shaken as the 0.2 N acid is run in from the burette.

Sometimes it is desirable to use a similar technique for the phenolphthalein end-point also. In this case a tube of urine filtrate to which no phenolphthalein is added serves as a standard.

We have found that as the final end-point with tropeolin OO is approached comparison of colors is somewhat facilitated by placing the two tubes side by side in a comparator of the form described by Dernby and Avery, although with practice the end-point may be located within 0.1 cc. by merely holding the tubes together as described above.

#### *Calculation.*

From the volume of 0.2 N HCl used to titrate from the end-point of phenolphthalein to that of the tropeolin OO, the amount, usually 0.7 cc., is subtracted which is utilized in a similar titration of a control determination in which water is substituted for the urine. The volume of 0.2 N HCl thus corrected represents the approximate organic acid content of the urine sample, plus the creatine and creatinine, and an amount of amino-acids ordinarily negligible.

In order to calculate the results in terms of cc. of 0.1 N organic acid per liter, the figure representing the cc. of 0.2 N HCl used in the titration is multiplied by 80 (by  $\frac{1,000}{25} = 40$  in order to transfer figure from 25 cc. to 1,000 cc. of urine, and by 2 to change from 0.2 N to 0.1 N terms).

*Correction for Creatinine.*—A 0.1 M solution of creatinine (11.32 mg. per cc.) titrates in the above determination as a 0.1 N solution of organic acid. Therefore, in order to correct for the creatinine, the cc. of 0.1 N organic acid per liter calculated from the above titration may be diminished by

$$\text{mg. creatinine per liter urine or by } \frac{\text{mg. creatinine N per liter urine}}{11.32} = 4.2$$

The simplest way is to subtract the creatinine correction directly from the cc. of 0.2 N acid used in the titration, and multiply the difference by 80. In this case the correction is  $\frac{1}{80}$  as great as the above;

i.e., cc. correction =

$$\text{mg. creatinine per liter urine} \text{ or } \frac{\text{mg. creatinine N per liter urine}}{906} \cdot \frac{336}{336}$$

*Example.—*

	cc.
0.2 N HCl used in titration.....	7.6
Correction found in blank analysis.....	0.7 cc.
Creatinine correction for 500 mg. creatinine N per liter urine.	
Correction = $\frac{500}{336}$ cc. = .....	1.2 "
Total correction.....	1.9 cc.
Corrected titration figure = 7.6 - 1.9.....	5.7
0.1 N organic acid per liter = $80 \times 5.7$ .....	456.0

Tropeolin OO was preferred by us as indicator for the final endpoint. In neutral solution it gives nearly the same yellow color as urine, but so much more intense that a water solution of 0.002 N hydrochloric acid with the indicator can be used as a color standard without the use of a comparator. Very dark urines may need greater dilution, but such are not often encountered. Another advantage of this indicator is that its maximum acid color is not reached even at pH 2.7, so that if too much HCl is added in the titration the solution becomes redder than the standard. This particular advantage is possessed in much less degree by the three indicators mentioned below as alternatives.

Other indicators that may be used are methyl orange, tetrabromo-phenolsulfonephthalein (bromophenol blue, Clark and Lubs), and dimethylaminoazobenzene. To some eyes the color change of one of these dyes may be more readily detected than that of tropeolin OO. The two azo dyes are not much different in color from tropeolin OO, both changing from yellow to red, but the bromophenol blue turns from blue to a clear yellow on acidifying, and affords a very different alternative. To the authors the tropeolin OO end-point appeared the most satisfactory, however.

*Theoretical Basis of Method.*

The method is based on the following previously known facts:

1. Relatively little strong mineral acid is required to change the hydrogen ion concentration of a water solution from  $10^{-8}$  to  $2 \times 10^{-8}$  if the only electrolytes present are alkali salts of strong acids, such as sulfates and chlorides.

2. If the salt of a weak acid is present, however, the addition of nearly a full molecule of hydrochloric acid for each molecule of such salt is necessary in order to cause the above change in hydrogen ion concentration. The organic acids known to occur in normal and pathological urines, in amounts sufficient to be quantitatively significant in the total acid excretion of the body, belong to the class of weak acids whose salts behave in the above manner.

3. The only mineral acids found in significant amounts in urine which belong to the class of weak acids, and therefore form salts which show the above behavior, are phosphoric and carbonic acids.

4. Very weak bases form salts which behave like those of the weak acids. Creatinine is titrated almost quantitatively in changing the hydrogen ion concentration from  $10^{-8}$  to  $2 \times 10^{-3}$ , and creatine to about 60 per cent. Aside from the traces of amino-acids, these appear to be the only bases of this kind present in considerable amount in human urine.

*Effect of the Different Organic Acids of the Urine on the Titration.*

The titration figure obtainable by titrating between two hydrogen ion concentrations a solution containing the salt of an acid of a known dissociation constant may be calculated as follows:

From the law of mass action:

$$(1) H^+ = k \frac{HA}{A'}$$

$H^+$  = hydrogen ion concentration in terms of normality.

$A'$  = anion of acid.

$k$  = dissociation constant of the acid.

$HA$  = free, undissociated acid.

$BA$  = salt of the acid.

$\lambda$  = degree of dissociation of the salt into  $Na$  and  $Ac.$

When the salt of the acid is present, and dilutions are of the magnitudes used in titrations (0.1 to 0.01 M) the equation becomes practically

$$(2) \quad H^+ = \frac{k}{\lambda} \times \frac{HA}{BA}$$

As  $\lambda$  in the high dilutions encountered approaches unity, it may in approximate calculations be neglected.

Equation 1 may then be expressed as

$$(3) \quad H^+ = k \times \frac{HA}{BA} \text{ or } \frac{HA}{BA} = \frac{H^+}{k}$$

For acetic acid  $k = 1.8 \times 10^{-5}$ .

When pH equals 8, or  $H^+ = 1 \times 10^{-8}$ , we therefore have in the case of acetic acid  $\frac{HC_2H_3O_2}{BC_2H_3O_2} = \frac{10^{-8}}{1.8 \times 10^{-5}} = \frac{1}{1,800}$ . One part in 1,801 parts, or 0.05 per cent, of the acid is free.

When pH = 2.7,  $H^+ = 2 \times 10^{-3}$ , and we have  $\frac{HC_2H_3O_2}{BC_2H_3O_2} = \frac{2 \times 10^{-3}}{1.8 \times 10^{-5}} = \frac{200}{1.8}$ .

At a pH of 2.7, therefore,  $\frac{200}{201.8}$ , or 99.2 per cent, of the acid is free.

Changing the hydrogen ion concentration of an acetate solution from the slightly alkaline reaction of  $10^{-8}$  N (or a pH of 8) to the acid reaction of  $2 \times 10^{-3}$  (pH = 2.7) approximately the reaction of 0.002 N HCl therefore requires an amount of HCl equal in molecular equivalents to 99.15 per cent of the total acetate present.

For the different acids which occur or may occur in human urine, the values in Table I are calculated. The values of the constants are for 25° unless otherwise indicated.

Comparison of the results so calculated with those experimentally obtained in titrating solutions of some of these acids is satisfactory, as shown in Tables III, IV, and VI of the experimental part of this paper. The conclusion seems justified that the titration as carried out estimates certainly over 90 per cent of the organic acids of the urine, and presumably over 95 per cent, since a higher titration value

by 3 or 4 per cent is obtained for those acids excreted as ammonium salts. The data for carbonic and phosphoric acids indicate the necessity for their removal before the organic acids are titrated.

TABLE I.  
*Calculated Titration Values of Weak Acids of the Urine.*

Acids.	Dissociation constant.	Acid free at		Calculated proportion of acid determined by titrating from pH 8 to pH 2.7.
		$H^+ = 10^{-8} N$ pH = 8	$H^+ = 2 \times 10^{-3}$ pH = 2.7	
		per cent	per cent	per cent
<i>Organic.</i>				
Uric*	$1.5 \times 10^{-6}$	0.5	99.9	99.4
Acetic†	$1.8 \times 10^{-5}$	0.0	99.2	99.2
$\beta$ -hydroxybutyric‡	$2.0 \times 10^{-5}$	0.0	99.0	99.0
Lactic†	$1.4 \times 10^{-4}$	0.0	93.5	93.5
Acetoacetic‡	$1.5 \times 10^{-4}$	0.0	93.1	93.1
Citric§.	$2.0 \times 10^{-4}$	0.0	91.0	91.0
Formic*	$2.1 \times 10^{-4}$	0.0	90.6	90.6
Hippuric†	$2.2 \times 10^{-4}$	0.0	90.2	90.2
<i>Mineral.</i>				
$H(NaHPO_4) \parallel$	$2.0 \times 10^{-7}$	2.5	100	97.5
$H(HCO_3) \parallel$	$3.5 \times 10^{-7}$	4.2	100	95.8

\* His and Paul.

† Ostwald.

‡ Henderson and Spiro.

§ Shown by Amberg and McClure to occur in amounts equivalent to 60 to 70 cc. of 0.1 N acid in a normal 24 hour urine. The titration value for citric acid given in Column 5 is that directly determined by Sörensen. The constant is estimated from it.

|| Sörensen.

¶ Kendall.

### *Effect of Weak Bases of the Urine on the Titration.*

The amount of strong acid required to change the pH of a solution of a weak base from 8 to 2.7 may be calculated from the dissociation constant  $K_b$ .

$$K_b = OH' \times \frac{\text{salt of base}}{\text{free base}} = \frac{10^{-14}}{H^+} \times \frac{\text{salt of base}}{\text{free base}}$$

The "salt of base" represents the amount combined with acid. At pH 8, therefore, salt of base, or  $\frac{\text{acid combined with base}}{\text{free base}} = K_b \times \frac{10^{-8}}{10^{-14}} = K_b \times 10^6$ . At pH 2.7, or  $H^+ = 2 \times 10^{-8}$ , the ratio is  $K_b \times \frac{2 \times 10^{-8}}{10^{-14}} = 2 K_b \times 10^{11}$ . The difference between the acid bound by a given base at pH 8 and that bound at pH 2.7 represents the amount required to titrate between the two points. Table II contains a list of the weak bases of the urine, with their constants and the propor-

TABLE II.  
*Calculated Titration Values of Organic Bases of the Urine.*

Base.	Basic dissociation constant. $K_b$	Base free at		Proportion estimated by titrating with HCl from $H^+ = 10^{-8}$ to $H^+ = 2 \times 10^{-8}$	
		$H^+ = 10^{-8}$	$H^+ = 2 \times 10^{-8}$	Calculated.	Observed.
Urea*	$0.0015 \times 10^{-11}$	100	99.7	0.3	0.2
Creatinine†	$1.81 \times 10^{-11}*$	100	24.0	76.0	99
Creatine‡	$3.57 \times 10^{-11}*$	100	12.3	87.7	60
Ammonia‡	$1.5 \times 10^{-5}†$	6.2	0.0	6.2	5.3-6.0

\* Walker and Wood.

† Measured at 40°, Wood.

‡ Noyes, Kato, and Sosman.

tion of an equivalent of HCl required to titrate each from pH 8 to pH 2.7, calculated as above indicated. The constants are from data obtained at 25°, except for creatine and creatinine. In Column 5 results are brought forward from Table V, showing the amounts of HCl bound by the different bases in the titration, as determined experimentally.

Urea is, both by observation and calculation, practically without effect on the results of the titration, even when the urea concentration is at the maximum observed in human urine.

The available data on the  $K_b$  of creatine and creatinine do not yield calculated results corresponding so closely with those experimentally

obtained as do the data on the other substances requiring consideration. The divergence is perhaps due to the fact that Wood's values for  $K_b$  of creatine and creatinine were determined at 40°, while the titration is performed at 20°. It is evident, however, that practically all the creatinine is titrated as organic acid. The amount of this substance excreted varies between 13 and 27 mg. per kilo of body weight per 24 hours (Folin, 1905). The mean, 20 mg., would neutralize 1.8 cc. of 0.1 N acid per kilo or 108 cc. for a 60 kilo individual.

Creatine when present titrates to about 60 per cent as an organic acid; but it is excreted by adults only in conditions involving rapid autolysis of muscle tissue, and would therefore not, as a rule, require consideration.

Ammonia is titrated to the extent of 5 to 6 per cent, but the actual effect of the presence of organic acids as ammonium rather than fixed alkali salts is to make the results of the titration with most of the acids approximate more closely the theoretical values, as shown in Table IV. The ammonium salts of the organic acids titrate 2.3 to 4.6 per cent more completely than the sodium salts, not 6.2 per cent more completely, as would be theoretically expected, and as is approximately realized for the ammonium salts of hydrochloric and sulfuric acids. The observed positive ammonia error is such as to make the results obtained with all but the weakest organic acids approximate more closely to 100 per cent than the results obtained in the absence of ammonia. The tendency of the ammonia error to correct the opposite error in the organic acid titration is enhanced by the fact that ammonia and organic acid excretion tend to run parallel, particularly when acid excretion is abnormally high, as in diabetic acidosis. For the reasons, therefore, that the ammonia correction is not great and is of a nature actually to diminish, as a rule, the other error in the determination, it has seemed not only simpler but better to attempt no correction for it in urine analyses.

#### *Effect of Amino-Acids on the Titration.*

Amino-acids if present in large amount would be disturbing factors, as at an  $H^+$  of  $2 \times 10^{-3}$  they bind with their  $NH_2$  groups considerable amounts of acid. Glycocoll, which does not differ much from

the other monoamino-acids in this respect, binds about  $\frac{1}{2}$  molecule of HCl at this H<sup>+</sup>. The amount is calculated as follows:

The acid constant for glycocoll is  $3.4 \times 10^{-10}$ , the basic constant  $2.9 \times 10^{-12}$ , as calculated by Winkelblech from conductivity measurements. From the acid constant we have by calculating as above:

$-\text{COOH}$ free at H <sup>+</sup> = $10^{-8}$ N	$-\text{COOH}$ free at H <sup>+</sup> = $2 \times 10^{-8}$ N	Proportion of COOH group estimated by titration from H <sup>+</sup> = $10^{-8}$ to H <sup>+</sup> = $2 \times 10^{-8}$
per cent	per cent	per cent
96.7	100	3.3

The function of the NH<sub>2</sub> group is similarly calculated from the basic constant,  $K_b = 2.9 \times 10^{-12} = (\text{OH})' \times \frac{\text{glycine chloride}}{\text{free glycine}}$  or

$$\frac{\text{COOH-CH}_2\text{-NH}_2}{\text{COOH-CH}_2\text{-NH}_2\text{HCl}} = \frac{(\text{OH})'}{2.9 \times 10^{-12}} = \frac{10^{-14}}{\text{H}^+ \times 2.9 \times 10^{-12}} = \frac{10^{-2}}{\text{H}^+ \times 2.9}.$$

From these values we calculate:

$-\text{NH}_2$ free at H <sup>+</sup> = $10^{-8}$ N	$-\text{NH}_2$ free at H <sup>+</sup> = $2 \times 10^{-8}$ N	Proportion of NH <sub>2</sub> group estimated by titration with HCl from H <sup>+</sup> = $10^{-8}$ to H <sup>+</sup> = $2 \times 10^{-8}$
per cent	per cent	per cent
100	63.3	36.7

The total consumption of HCl by both COOH and NH<sub>2</sub> groups in the titration should be, according to the above calculation,  $0.03 + 0.367 = 0.40$  molecule of HCl per 1 molecule of glycine present. The actual amount observed by Sörensen was 0.385 molecule.

The other monoamino-acids apparently bind similar amounts of HCl. The constants for leucine and alanine were determined by Winkelblech as follows: leucine,  $K_a = 3.1 \times 10^{-10}$ ,  $K_b = 2.7 \times 10^{-12}$ ; alanine,  $K_a = 9.0 \times 10^{-10}$ ,  $K_b = 3.8 \times 10^{-12}$ . According to these, leucine would require in the titration 0.38 molecule of HCl; alanine 0.36, nearly the same as glycocoll. The results in Table VII for the mixture of all the monoamino-acids obtained from casein are in the same neighborhood (44 per cent).

The amino-acid nitrogen constitutes 1 to 2 per cent of the total urinary nitrogen (Van Slyke, 1913-14; Henriques). On a daily excretion of 14 gm. of nitrogen, 2 per cent would indicate 200 cc. of 0.1 N amino-acids. The neutralizing power of such an amount of amino-acids in the titration would be about 80 cc. of 0.1 N hydrochloric acid.

Our knowledge of the nitrogenous constituents of the urine indicates the presence of no weak bases, aside from those discussed, in quantities sufficient to affect markedly the organic acid titration under discussion, and the nitrogenous excretory products have been so thoroughly studied that it is unlikely that any quantitatively important substances with definitely basic properties have been overlooked.

It therefore appears that in titrating the 24 hour urine of an adult of average size for organic acids, as described in this paper, about 100 cc. of the 0.1 N organic acid estimated is in reality due to creatinine and creatine, 80 cc. or less to amino-acids, and the remainder to organic acids.

#### EXPERIMENTAL.

*Titration of Organic Acids in Water Solutions.*—A 20 cc. portion of each acid, of approximately 0.1 N concentration, was titrated in a 100 cc. test-tube with either 0.1 N sodium hydroxide or 0.1 N ammonium hydroxide to neutrality with 0.5 cc. of 1 per cent phenolphthalein. 1 cc. of 0.1 per cent tropeolin OO was then added, and the solution titrated back with 0.2 N HCl to pH 2.7, using 0.002 N HCl solution as standard. The results are given in Tables III and IV.

*Titration of Weak Bases in Water Solutions.*—Solutions of the bases in 25 cc. portions were brought to pH 8 by addition of 0.1 N NaOH or 0.2 N HCl until a barely visible pink color was reached; then tropeolin OO was added and the solution titrated to pH 2.7. The results are given in Table V.

*Effect of Concentration of Phenolphthalein on Its End-Point in Presence of Ammonium Salts.*—The concentration of phenolphthalein to some extent affects the pH at which the pink color is just visible. If there is but little indicator present a greater part of it must be in the colored form to give a perceptible pink than when the total amount of indicator is greater. Consequently the amount of extra alkali

TABLE III.  
*Titration of Sodium Salts of Organic Acids.*

Acid.	(A) 0.1 N NaOH to neutralize acid to phenolphthalein.	(B) 0.2 N HCl to titrate back to pH 2.7 with troponin OO.	(C) Average 0.2 N HCl corrected for blank.	(D) Organic acid determined. $\frac{200 \text{ (C)}}{(A)}$	Organic acid theoretically titratable from pH 8 to pH 2.7 (from Table I).
	cc.	cc.	cc.	per cent	per cent
Blank.....	0.1	0.50	0.00		
Acetic.....	20.00	10.60 10.50	9.95	99.5	99.4
Citric.....	19.86	9.30 9.35	8.88	89.4	91.0
Lactic.....	20.28	9.90 9.90	9.40	92.7	93.5
Hydrochloric.....	20.00	0.70	0.20	1.0	

TABLE IV.  
*Titration of Ammonium Salts of Organic Acids.*

Acid.	(A) 0.1 N acid present.	(B) 0.1 N NH <sub>4</sub> OH to neutralize acid to phenolphthalein at pH 8.	(C) 0.2 N HCl to titrate back to pH 2.7.	(D) Average 0.2 N HCl corrected for 0.5 cc. blank.	(E) Proportion of organic acid determined. $\frac{200 \text{ (D)}}{(A)}$	Proportion of NH <sub>4</sub> salt theoretically titratable; i. e., that for acid calculated in Table I + 6.2 per cent for NH <sub>4</sub> present.	Differences between average percentage of Na salt and NH <sub>4</sub> salt titrated.
	cc.	cc.	cc.	cc.	per cent	per cent	per cent
Acetic.....	19.68	20.51	10.70	10.25	104.1	105.6	4.6
	19.68	20.47	10.80				
Citric.....	21.04	21.55	10.30	9.79	93.0	97.2	3.5
	21.04	21.51	10.27				
Lactic.....	20.06	20.96	10.03	9.50	94.7	99.7	2.3
	20.06	20.96	10.03				

required to make a solution of an ammonium salt show pink with phenolphthalein is somewhat dependent on the amount of indicator used. This is shown by the results in Table VI. It is desirable to use in performing the titrations 0.5 cc. of 1 per cent phenolphthalein

TABLE V.

*Observed Behavior of Weak Bases when Titrated from pH 8 to pH 2.7.*

Base.	Amount present in the 25 cc. of solution titrated.		0.2 N HCl required in titrating from pH 8 to pH 7.	Proportion of base titrated.  per cent	Proportion of base calculated as titratable from dissociation constant (Table II).  per cent
	gm.	cc. 0.2 N			
Urea.....	1.000	83.3	0.1	0.12	0.3
Creatine.....	0.200	7.6	4.1	60.0	87.7
Creatinine.....	0.100	4.41	4.32	97.8	76.0
	0.200	8.83	8.80	99.7	
Monoamino-acid from casein.....	0.100	7.37*	3.25	44.2	36.0-40.0 for glycine, leucine, and alanine.
	0.200	14.63	6.37	43.5	
	0.200	14.63	6.29	43.0	
Ammonia (as · (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ).....		12.50	0.67	5.4	6.2
		12.50	0.75	6.0	
Ammonia (as NH <sub>4</sub> Cl). .		9.82	0.53	5.4	6.2
			0.52	5.3	

\* Calculated on a nitrogen content of 10.3 per cent. The preparation was made by hydrolyzing casein with sulfuric acid, precipitating the bases with phosphotungstic acid, and concentrating the filtrate to dryness under reduced pressure after the phosphotungstic and sulfuric acids had been removed.

TABLE VI.

*Effect of Phenolphthalein Concentration on End-Point in Presence of Ammonium Salts.*

0.05 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .	1 per cent phenol- phthalein.	0.1 N NaOH to turn pink to phenol- phthalein.	0.2 N HCl to change from phenolphthalein end-point to pH 2.7.		Proportion of ammonia titrated from phenol- phthalein end-point to pH 2.7.
			Uncorrected.	Minus 0.5 cc. for correction.	
cc.	cc.	cc.	cc.	cc.	per cent
25	0.1	0.85	1.42	0.92	7.3
25	0.2	0.65	1.36	0.86	6.9
25	0.5	0.45	1.20	0.70	5.6
25	1.0	0.45	Too cloudy with precipitated phenolphthalein to titrate.		

solution, as directed, rather than the indefinitely measured drop or two which suffices in ordinary titrations.

*Titration of Known Amounts of Organic Acids Added to Urine.*—100 cc. portions of a mixed sample of normal urine were mixed with portions of 25, 50, and 100 cc. respectively of acetic or lactic acid. Each

TABLE VII.  
*Titration of Organic Acids Added to Urine.*

Organic acid added.	0.1 N organic acid added to 100 cc. urine.	0.2 N HCl used in duplicate titrations of 25 cc. urine filtrate.	Average titration figure minus that for urine alone.	0.1 N added organic acid per liter diluted urine.		Proportion of added organic acid determined.
				Found.	Added.	
Acetic.	cc.	cc.	cc.	cc.	cc.	per cent
	0	3.00 3.00				
	25	4.55 4.53	1.54	123	125	98.4
	50	6.20 6.15	3.17	253	250	101.2
Lactic.	100	9.15 9.10	6.13	490	500	98.0
	0	2.87 2.87				
	25	4.25 4.20	1.36	109	117*	93.2
	50	5.50 5.60	2.68	214	236*	90.7
	100	8.30 8.25	5.41	432	472*	91.6

\* The 0.1 N lactic acid used in this experiment had the factor 0.945.

mixture was then diluted to 200 cc., and 100 cc. portions were treated as previously described for determination. The results are given in Table VII. The results are essentially the same as those obtained with acetic and lactic acids in pure water solutions.

TABLE VIII.  
*Excretion of Organic Acids with Creatinine Correction.*  
Data from hospital patients.

Subject.	Weight.	Condition.	Period.	Volume.	Creatinine N.	Urine excretion.		0.1 N organic acid content.	
						Duplicates.	Average minus 0.6 cc. correction for blank.	Total.	Per kilo.
Z	60	Myocarditis, decompensation on admission.	12 (day) 12 (night) 24	cc. 658 946 1,604	gm. 0.240 0.261 0.501	cc. 0.1 M 57 62	cc. 7.0, 6.9 4, 3, 4.1	cc. 334 272	cc. 277 210
O	60	Myocarditis, some decompensation.	12 (day) 12 (night) 24	707 744* 1,451	0.223 0.216 0.439	cc. 53 51 104	5.1, 5.25 5, 0, 5.0 4, 4.0	cc. 487	cc. 10.5
C	55	Chronic aortic endocarditis.	12 (day) 12 (night) 24	242 332 574	0.141 0.195 0.336	cc. 34 46 80	15.1, 15.3 12.8, 12.9 12.25	cc. 258 262 520	cc. 205 211 416
D	62	Chronic myocarditis with decompensation.	12 (day) 12 (night) 24	647 750 1,397	0.226 0.196 0.422	cc. 54 47 101	5.6, 5.7 4.4, 4.3 3.75	cc. 282 325 607	cc. 248 279 527
H	50	Orthostatic albuminuria.	12 (day) 12 (night) 24	364 380 744	0.091 0.091 0.182	cc. 22 22 44	5.55, 5.55 5.25, 5.10 4.48	cc. 144 136 486	cc. 122 114 385

*Organic Acid Excretion by Individuals with Normal Metabolism.*

The data given are sufficient only to indicate the usual excretion of organic acids; the possible normal variations, particularly under unusual conditions, may be greater. The figures of Table VIII are from afebrile heart patients, with apparently normal metabolism.

TABLE IX.

*24 Hour Excretion of Organic Acids by Normal Young Men.*

Subject.	Weight. kg.	24 hour urine.			
		Volume. cc.	0.1 N organic acids uncorrected for creatinine.*	cc. per kg.	Total N. gm.
Ce . . . . .	54.4	1,000	492	9.0	9.3
Dy . . . . .	68.0	1,650	657	9.8	11.5
E . . . . .	68.0	975	583	8.5	11.7
Fr . . . . .	62.1	1,500	531	8.5	13.2
H . . . . .	68.0	1,150	412	6.1	7.8
Sh . . . . .	56.6	1,500	453	8.0	10.0
K . . . . .	68.4	1,000	490	7.2	8.7
Sp . . . . .	57.2	1,400	521	9.1	9.0
Fe . . . . .	82.6	1,100	748	9.1	15.5
Dn . . . . .	87.0	1,300	493	5.7	13.2
Ch . . . . .	56.2	1,100	420	7.5	11.2
K . . . . .	61.2	700	499	8.2	10.0
Ck . . . . .	56.6	1,300	547	9.7	12.1
Average . . . . .				8.2	

\* The creatinine correction would reduce the total organic acid figure by about 2 cc. per kilo.

The day periods are from 6 a.m. to 6 p.m., the night periods from 6 p.m. to 6 a.m. The data of Table IX are from a series of healthy young men. The figures indicate that the usual excretion of organic acids uncorrected for creatinine varies from about 280 to 750 cc. of 0.1 N acid per 24 hours, or from 5.6 to 11 cc. per kilo of body weight. The creatinine correction reduces the figures to 240 to 600 and 4.7 to 9.6 cc. respectively.

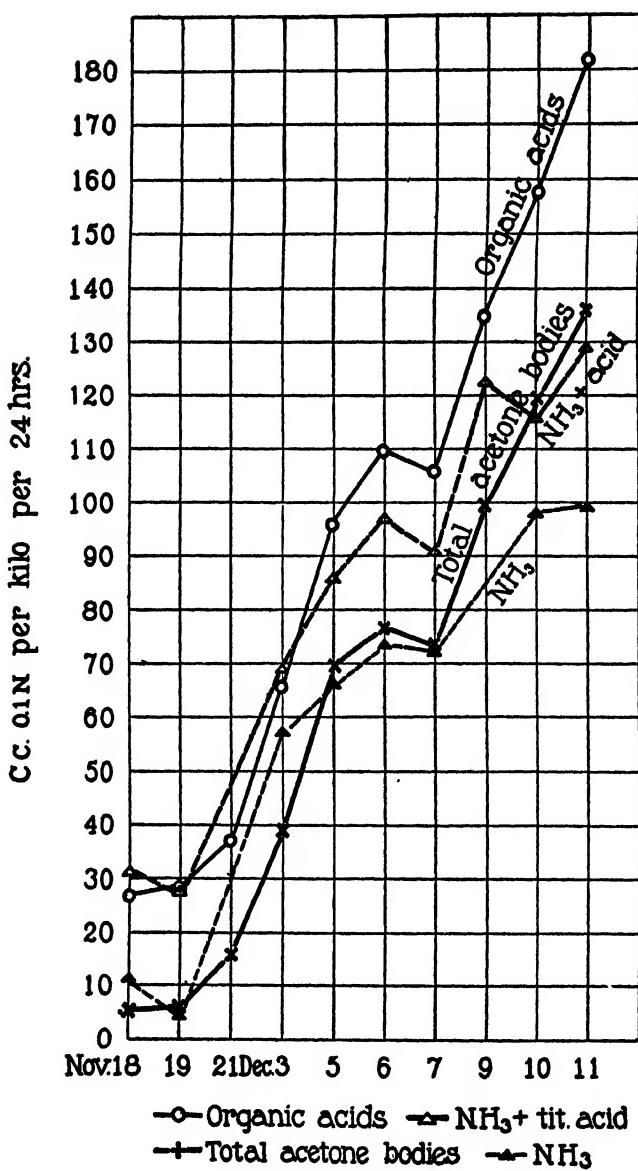


FIG. 1. Excretion in a case of diabetic acidosis.

*Comparison of Total Organic Acid Excretion with Acetone Bodies Excretion in Diabetes.*

The data given in Fig. 1 were obtained with the only case of diabetic acidosis which we have studied since the organic acid titration method has been available. Although all the data are from one case, they nevertheless represent every stage of diabetic acidosis, from the time when it was slight, with little ketonuria, up to the point of coma, with tremendous ketonuria. The patient was a child of 2 years,

TABLE X.  
*Organic Acid Excretion in a Non-Fatal Case of Methyl Alcohol Poisoning.*

Date.	$\text{CO}_2$ bound as bicarbon- ate by 100 cc. of plasma.	Excretion per liter urine.						
		Creatine.	Creatinine.	Total 0.1 N organic acids.*	0.1 N acetone bodies.	0.1 N lactic acid.	0.1 N formic acid.	Undeter- mined 0.1 N organic acids.
1919	cc.	gm.	gm.	cc.	cc.	cc.	cc.	cc.
Nov. 24...	36.4	0.202	0.558	2,042		173	274	1,595
" 25...	36.0	0.283	1.000	2,076	481	83		1,512
" 26...	86.2	0.535	0.800	1,377	143		30	1,074
" 29...	76.7	0.300	0.590	262			130	
Dec. 1...		0.180	0.538	129				
" 2...		0.137	0.557	141				
" 3...				220†				
" 11...		0.105	0.378	86				
" 17...		0.024	0.476	138				
" 20...								

\* Corrected for creatine and creatinine.

† " " " " " estimated not determined.

weighing 8 kilos. The organic acid figures recorded in Fig. 1 are not corrected for creatine and creatinine, so that they are higher than, though parallel to, the actual organic acid excretion. The "total acetone bodies,"  $\beta$ -hydroxybutyric acid, acetoacetic acid, and acetone were determined by the gravimetric method of Van Slyke (1917), the ammonia as described by Van Slyke and Cullen, and the titratable acid by the method of Folin (1903).

It is evident from the chart that the organic acids of the urine, determined by the technique outlined above, paralleled the acetone

body excretion with a high degree of accuracy through all stages of the acidosis, the parallelism being more accurate than that of the ammonia, or even the ammonia plus titratable acid.

It appears that the rise above the normal output in organic acid excretion may be used as an approximate measure of the acetone body excretion in diabetes, the determination of organic acids being as simple as that of ammonia and less influenced by other factors, such, in particular, as alkali administration.

*Organic Acid Excretion in Methyl Alcohol Poisoning.*—The data of Table X illustrate an acidosis caused by organic acids other than the familiar acetone bodies. The data represent some preliminary work on methyl alcohol poisoning and are inserted here only for their interest in illustrating a hitherto unfamiliar type of acidosis.

#### SUMMARY.

The organic acids present both free and as salts in urine are estimated by titrating between the hydrogen ion concentrations represented by pH 8 and pH 2.7 respectively, after removal of phosphates and carbonates by means of calcium hydroxide. It appears that the titration represents between 95 and 100 per cent of the organic acids present. It also includes weak bases whose dissociation constants fall within a range in the neighborhood of  $10^{-11}$ , but of this class only creatinine, and at times creatine, appear to be present in significant amounts in human urine.

The average 24 hour excretion of organic acids in thirteen healthy young men was, per kilo of body weight, 8.2 cc. of 0.1 N acid uncorrected for creatinine, or approximately 6 cc. corrected for creatinine; the extreme range was from 5.7 to 9.8 cc. uncorrected for creatinine. There appears to be little difference between day and night periods in rate of organic acid excretion.

Data from cases of methyl alcohol poisoning and diabetes respectively are given as examples of acidosis due to organic acids of different types. In the case of methyl alcohol poisoning part of the total organic acid excretion was due to formic, lactic, and hydroxybutyric acids, but the greater part to acids of unknown nature.

In the case of diabetes, which progressed to coma, the rise in acetone body excretion was accurately paralleled by the rise in the titrated organic acids. The parallelism was so close as to indicate the probabilities (1) that organic acids other than the acetone bodies are not excreted in significant amounts in diabetic acidosis, and (2) that the easily performed organic acid titration may be used for approximate estimation of the acetone bodies in diabetic urine.

#### BIBLIOGRAPHY.

- Amberg, S., and McClure, W. B., *Am. J. Physiol.*, 1917, xliv, 453.  
Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.  
Dernby, K. G., and Avery, O. T., *J. Exp. Med.*, 1918, xxviii, 345.  
Folin, O., *Am. J. Physiol.*, 1903, ix, 265; 1905, xiii, 66.  
Henderson, L. J., and Spiro, K., *J. Biol. Chem.*, 1909, vi, p. xxxix.  
Henriques, V., *Z. physiol. Chem.*, 1909, lx, 1.  
His, W., and Paul, T., *Z. physiol. Chem.*, 1900-01, xxxi, 1.  
Kendall, J., *J. Am. Chem. Soc.*, 1916, xxxviii, 1480.  
Noyes, A. A., Kato, Y., and Sosman, R. B., *Z. physik. Chem.*, 1910, lxxiii, 1.  
Ostwald, W., *Z. physik. Chem.*, 1889, iii, 173.  
Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.  
Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, xvi, 125; 1917, xxxii, 455.  
Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211; 1916, xxiv, 117.  
Walker, J., and Wood, J. K., *J. Chem. Soc.*, 1903, lxxxiii, 484.  
Winkelblech, K., *Z. physik. Chem.*, 1901, xxxvi, 546.  
Wood, J. K., *J. Chem. Soc.*, 1903, lxxxiii, 568.



## DETERMINATION OF THE FIBRIN, GLOBULIN, AND ALBUMIN NITROGEN OF BLOOD PLASMA.

By GLENN E. CULLEN AND DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 24, 1920.)

An investigation into the distribution of the plasma nitrogen, which formed part of a study of the fate of the protein digestion products, led us to develop a technique for the determination of the plasma proteins which gives consistent results and requires no special apparatus. It is based entirely on Kjeldahl determinations, of which the following four are required: (1) total nitrogen of the plasma, (2) fibrin nitrogen, (3) filtrate nitrogen (filtrate, containing albumin and non-protein nitrogen, obtained after precipitating the globulins by half saturation with ammonium sulfate), and (4) non-protein nitrogen.

In the technique finally arrived at the fibrin was precipitated by calcium chloride under definite conditions from plasma containing 0.5 per cent of potassium oxalate, was washed free from other nitrogenous substances, and determined by Kjeldahl.

The globulin was precipitated (together with the fibrin) by the usual half saturation with ammonium sulfate. The nitrogen of the filtrate was determined by Kjeldahl, after removal by distillation of the ammonia of the ammonium sulfate. For the distillation it was found necessary to standardize the conditions accurately in order to make the removal quantitative and at the same time avoid splitting off labile nitrogen from proteins. The non-protein nitrogen was determined on a separate sample of plasma.

The fibrin, globulin, and albumin are calculated as follows:

Fibrin N, determined directly.

Globulin N = Total N - (filtrate N + fibrin N).

Albumin N = Filtrate N - non-protein N.

There are two steps in the determination of the albumin and globulin contents of plasma; the first is the separation of the two proteins, or groups of proteins, by precipitation of the globulin with salt, either saturated magnesium sulfate, or half saturated ammonium sulfate. Robertson has reviewed the work on globulin precipitation, and is convinced that ammonium sulfate is the most satisfactory salt for the purpose. We have, therefore, utilized ammonium sulfate precipitation from the start, and since the results have been uniformly consistent have not experimented with other globulin precipitants.

The second step is the determination of the proteins after they have been separated. By different authors this has been done by weighing, by nitrogen determination after dialysis to remove ammonium salts, by the nephelometric method, or by the use of the refractive indices of the proteins as developed by Reiss and by Robertson. The errors inherent in washing and weighing the globulin precipitate are too great to allow accurate results. With nephelometric determinations we have not been able to obtain the desirable degree of accuracy, and a proper refractometer was not available at the time the work was done. We consequently were led to develop a technique in which all the final determinations were made by the Kjeldahl method.

#### *Description of Methods.*

##### *Fibrin Determination.*

To 5 cc. of plasma, from blood to which 0.5 per cent of potassium oxalate has been added, add 150 cc. of 0.8 per cent NaCl and 5 cc. of a calcium chloride solution containing 2.5 gm. of anhydrous  $\text{CaCl}_2$  per 100 cc. Allow complete coagulation to occur (10 to 15 minutes) and filter through filter paper. Wash with 0.8 per cent NaCl five times, allowing each washing to remain in contact with fibrin for 10 minutes by closing the outlet of the funnel for that period. Transfer filter paper containing fibrin clot to Kjeldahl flask and add 20 cc. of sulfuric acid, 12 gm. of potassium sulfate, and a crystal of copper sulfate, and determine nitrogen in the usual manner.

*Albumin Determination.*

*Precipitation of Globulin.*—To 5 cc. of plasma add 20 cc. of water and 25 cc. of saturated ammonium sulfate solution, allow to stand over night, and filter through a dry filter.

*Removal of Sulfate Ammonia.*—Place 20 cc. of filtrate (= 2 cc. of plasma) in a 500 cc. Kjeldahl flask, add 300 cc. of 50 per cent alcohol, 3 gm. of MgO (Merck's reagent), and 1 cc. of white mineral oil. Distill until distillate gives a negative test with red litmus paper.

*Digestion of Residue.*—To residue add 25 cc. of concentrated  $H_2SO_4$ , 5 gm. of  $K_2SO_4$  (addition of more, with magnesium sulfate present, would cause bumping), and a small crystal of copper sulfate. Digest to a light brown color. Then wash flask down with a few cc. of water and add 10 cc. more of  $H_2SO_4$ . Continue digestion over a low flame for about 3 hours. Distill into N/14 HCl in the usual manner. Calculate nitrogen as "filtrate nitrogen."

$$\text{Albumin nitrogen} = \text{Filtrate nitrogen} - \text{non-protein nitrogen}.$$

*Total Plasma Nitrogen Determination.*

The total nitrogen determinations are carried out on 2 cc. of plasma by the regular Gunning-Kjeldahl method, using 20 cc. of concentrated  $H_2SO_4$ , 12 gm. of  $K_2SO_4$ , about 0.2 gm. of copper sulfate, and digesting 3 hours after clearing.

*Non-Protein Nitrogen Determination.*

The non-protein nitrogen is determined in the filtrate obtained by precipitation of the plasma protein in 9 volumes of 2.5 per cent trichloroacetic acid (Greenwald, 1915). A 50 or 100 cc. measuring flask is half filled with the trichloroacetic acid solution, to which 5 or 10 cc. of plasma are added. The flask is then filled to the mark with the trichloroacetic acid solution, and the contents are thoroughly mixed. After standing 1 hour the contents of the flask are filtered through a dry filter, the filtrate is measured, and transferred to a Kjeldahl flask. 20 cc. of  $H_2SO_4$ , 12 gm. of  $K_2SO_4$ , and a crystal of copper sulfate are added, and the nitrogen is determined in the usual manner.

On the basis of Greenwald's recent results (1918), it would seem slightly preferable to use 5 per cent rather than 2.5 per cent trichloroacetic acid. The differences introduced are so minute, however, that for the determination of the proteins they are not significant.

### *Correction for Reagents.*

It is necessary to determine the corrections for all the reagents. Our blanks averaged 0.46 cc. of N/14 HCl, a rather high value, but one constant for the given lot of reagents.

## EXPERIMENTAL.

### *Determination of Filtrate Nitrogen.*

In order to determine the albumin and non-protein plasma nitrogen in the filtrate from the globulin, it was necessary to find conditions for distilling off the ammonia of the ammonium sulfate without splitting off ammonia from any of the plasma proteins. In order to avoid such decomposition it was desirable to use in the distillation as weak an alkali as possible. Magnesium oxide, in former work on protein analyses (Van Slyke), had been found to be as mild an alkali as could be successfully used to drive off ammonia, and it proved to be suitable in this case also when used together with alcohol. It was found that the physical properties of the oxide were of importance. Tremendous bumping, resulting in broken flasks, took place with all but one brand of MgO. When Merck's reagent oxide was used with the addition of 1 cc. of white mineral oil, and the flask with its contents was frequently shaken until the boiling commenced, the distillation proceeded smoothly and without bumping.

*Distillation with Water.*—2 cc. of plasma and 10 cc. of saturated ammonium sulfate solution were diluted with 200 cc. of water in a 500 cc. Kjeldahl flask and an excess of magnesium oxide, 2 to 3 gm., was added (at 20°C. a half saturated ammonium sulfate solution contains 38 gm. per 100 cc. of the solution; 10 cc. would then require 1.7 gm. of MgO). The water and ammonia were distilled off. The nitrogen in the residue was then determined as outlined below.

In each case distillation was continued until moistened red litmus paper held in the distillate no longer turned blue at once. Actual cessation of ammonia distillation did not occur, because of a slight but continuous splitting off of ammonia from the proteins. Consequently if the litmus paper was held in the distillate for 2 minutes, an alkaline reaction could be obtained at any stage of the distillation. The end-point was therefore taken as the stage at which the distillate failed to turn litmus at once.

TABLE I.

*Albumin Determination. Removal of Ammonium Sulfate Nitrogen by Water Distillation in Presence of Magnesium Oxide.*

2 cc. plasma + 10 cc. saturated  $(\text{NH}_4)_2\text{SO}_4$  solution. Total nitrogen of plasma controlled on 2 cc. duplicates.

Method of concentration.	MgO	H <sub>2</sub> O used.	Final volume.	Proportion of plasma nitrogen recovered.
	gm.	cc.	cc.	per cent
Distillation from Kjeldahl flask.	2.3	400 in two portions.	50	98.3
" " "	2.3	400 " " "	50	97.8
" " "	2.3	400 " " "	50	96.0
" " "	2.3	400 " " "	50	97.8
" " "	2.3	400 " " "	50	96.0
" " "	2.3	400 " " "	50	96.0
" " "	2.3	400 " " "	50	93.0
" " "	2.3	400 " " "	50	94.0
" " "	2.3	400 " " "	50	92.0

It was found that frequently the ammonia was not completely removed by distilling nearly to dryness once; it was necessary to add a second 200 cc. of water and distill again. With two distillations all the sulfate ammonia was removed, but with it from 2 to 8 per cent of the plasma nitrogen was lost, apparently as the result of ammoniacal decomposition of the plasma proteins (Table I). Regulation of the rate of distillation, of the final volume, etc., all failed to prevent this loss.

*Distillation with Alcohol.*—In attempting to reduce both time and temperature of distillation, a mixture of alcohol and water was substituted for the water. A few of the results are given in Table II.

It is evident that with the use of 50 per cent alcohol, 99 per cent of the plasma nitrogen can be consistently recovered. One distillation only, taking 30 to 45 minutes, is required to drive off all ammonia. In studying the distribution of the plasma proteins following digestion, the method was tested several times on plasma from each of a dozen dogs. Between 99.8 and 98.5 per cent of the plasma nitrogen was invariably recovered.

TABLE II.

*Albumin Determination. Removal of Ammonium Sulfate Nitrogen by Distillation in Presence of MgO and Alcohol.*

2 cc. plasma + 10 cc. saturated  $(\text{NH}_4)_2\text{SO}_4$  solution.

MgO gm.	H <sub>2</sub> O cc.	95 per cent alcohol. cc.	Final volume. cc.	Proportion of plasma nitrogen recovered. per cent
3.5	150	150	25	99.6
3.0	150	150	25	99.2
2.5	150	150	15	99.6
3.0	150	150	Dry.	97.9
3.0	150	150	25	99.0
3.0	150	150	25	99.6
3.0	150	150	25	99.1
3.0	150	150	25	99.2
				99.0

### *Fibrin Determination.*

*Dilution of Plasma.*—If calcium is added to undiluted oxalated plasma, the entire mass jellies. If, however, the plasma is diluted with isotonic NaCl solution, the fibrin forms as a delicate membrane which contracts upon shaking or stirring to a small compact mass. Moreover, if the plasma is diluted ten- to thirtyfold the quantity of nitrogen in the solution adhering to the small clot is presumably much smaller than if the fibrin is whipped from undiluted plasma. In order to ascertain the best dilution, oxalated plasma was mixed with varying amounts of isotonic NaCl solution, which, to prevent globulin precipitation, was used instead of water. Preliminary experiments had shown that at least 2 molecules of calcium chloride should be added for each molecule of potassium oxalate present in the plasma.

The time required to complete the formation of the fibrin clot and the appearance of the clot were noted. Typical results are shown in Table III.

The clots formed when the plasma was diluted with 10 volumes of salt solution did not appear so satisfactory as those formed with either 20 or 30 volumes. There was no choice between 20 and 30 volumes,

TABLE III.

*Determination of Dilution Most Suitable for Plasma Fibrin Coagulation.*

1 cc. plasma diluted as indicated.

No.	Plasma. Oxalate concentration. <i>per cent</i>	0.8 per cent NaCl. <i>cc.</i>	Calcium added for recalcification.		Time required for complete clotting. <i>min.</i>	Coagulation observed.
			CaCl <sub>2</sub> solution. <i>percent</i>	Oxalate equivalent. <i>cc.</i>		
1	0.25	5	2.5	1	15	Not complete. Good. "
	0.25	10	2.5	1	15	
	0.25	20	2.5	1	15	
	0.25	30	2.5	1	15	
	0.25	40	2.5	1	15	
2	0.25	10	0.17	2	2	8.5
	0.25	20	0.17	2	2	8.5
	0.25	30	0.17	2	2	9
	0.25	10	0.17	4	4	7
	0.25	20	0.17	4	4	9.5
	0.25	30	0.17	4	4	9
	0.25	10	2.5	1	15	7
	0.25	20	2.5	1	15	8.5
1	1.0	10	0.48	5	15	7
	1.0	20	0.48	5	15	7
	1.0	30	0.48	5	15	7
	1.0	40	0.48	5	15	21

but with 40 volumes the clot appeared less satisfactory, and the time required for its formation was greater than with 20 or 30 volumes. The use of either 20 or 30 volumes of 0.8 per cent salt solution was, therefore, adopted.

*Permissible Range of Calcium Chloride Concentration.*—In order to determine the range of calcium concentration over which satisfactory

fibrin clot formation can occur, varying amounts of calcium chloride were added to a series of tubes each containing 1 cc. of plasma and 20 cc. of salt solution.

It is evident from Table IV that fibrin formation is complete if calcium is added in from two to twenty equivalents of the oxalate present. Allowing for a maximum oxalate concentration of 1 per cent,

TABLE IV.

*Influence of Concentration of Calcium Chloride on Fibrin Clot Formation.*

1 cc. plasma + 20 cc. 0.8 per cent NaCl recalcified in the presence of varying amounts of oxalate.

Oxalate concentration. per cent	Calcium added for recalcification.			Time required for complete clotting. min.
	CaCl <sub>2</sub> solution. per cent	cc.	Oxalate equivalent.	
1	0.5	0.5	0.37	∞
1	0.5	1	0.75	∞
1	0.5	2	1.5	9.0
1	0.5	4	3.0	5.0
1	2.5	1	3.7	5.5
1	2.5	2	7.5	6.5
1	2.5	4	15.0	11.0
0.25	2.5	0.25	3.7	7.0
0.25	2.5	0.5	7.5	7.5
0.25	2.5	1.0	15.0	7.5
0.25	2.5	1.5	22.5	9.0
0.25	2.5	2.0	30.0	>15
0.25	0.17	0.5	0.5	>30
0.25	0.17	1	1	>30
0.25	0.17	2	2	9
0.25	0.17	4	4	9

each cc. of plasma would require for an equivalent amount of calcium 0.2 cc. of 2.5 per cent CaCl<sub>2</sub>. 1 cc. of 2.5 per cent CaCl<sub>2</sub> solution would then contain five equivalents. If the oxalate concentration were 0.25 per cent, which is just sufficient to prevent coagulation, the use of an equal volume of 2.5 per cent CaCl<sub>2</sub> would mean that twenty equivalents of calcium had been used. This amount of CaCl<sub>2</sub> does not interfere with the clot formation, and provides for all probable concentrations of oxalate.

*Collection, Washing, and Nitrogen Determination of the Fibrin.*—It has been our experience that the determination of the nitrogen content of a protein is a more accurate measure of its mass than the method of drying to constant weight. The errors due to adherent substances or to partial decomposition during drying are thus eliminated. After testing varying conditions for the Kjeldahl digestion the following procedure was adopted. The filter paper with the washed fibrin clot was transferred to a 500 cc. Kjeldahl flask. 20 cc. of concentrated sulfuric acid, 12 gm. of potassium sulfate, and a small crystal of copper sulfate were added, and the mixture was digested for 3 hours after the clearing of the solution.

TABLE V.  
*Quantitative Test of Fibrin Method.*

5 cc. plasma, 0.25 oxalate; 0.8 per cent NaCl solution, CaCl<sub>2</sub>, and washing as indicated. Nitrogen determined by Kjeldahl method.

H <sub>2</sub> O cc.	0.8 per cent NaCl added.	Calcium added for recalcification.		Washed.	Fibrin N per 100 cc. gm.
		CaCl <sub>2</sub> solution. cc.	Oxalate equiv- alent. cc.		
100	2.5	5	15	0.8 per cent NaCl five times.	0.046
150	2.5	5	15	0.8 " " " "	0.044
150	2.5	10	32	0.8 " " " "	0.044
150	1.0	5	6	0.8 " " " "	0.042
150	1.0	10	12	0.8 " " " "	0.044
50	1.0	10	12	0.8 " " " "	0.044
150	1.0	10	12	H <sub>2</sub> O till chloride-free.	0.042
50	1.0	10	12	" " "	0.048
100	1.0	10	12	" " "	0.048

The fibrin clot was collected by filtration. In order to be sure that all traces of soluble proteins were removed from the clot, experiments were done to determine whether or not washing with NaCl only or with water was more satisfactory. Each washing was allowed to remain in contact with the clot for several minutes to allow time for diffusion. This was done by closing the outlet at the bottom of the funnel stem for the desired time.

It is evident from Tables V and VI that five washings with salt solution or washing with water until the filtrate is salt-free yields consistent results.

TABLE VI.

*Test of Different Methods of Washing Fibrin Clot.*

5 cc. plasma diluted to 150 cc. with 0.8 per cent NaCl, 5 cc. 2.5 per cent CaCl<sub>2</sub> solution added. Fibrin washed on filter paper as indicated. Nitrogen determined by Kjeldahl method.

0.8 per cent NaCl.	H <sub>2</sub> O	Each washing allowed to stand.	Fibrin N per 100 cc.
		min.	gm.
Five times.		10	0.077
" "		10	0.075
" "		10	0.073
" "		15	0.075
" "		30	0.079
Five times.		30	0.075
" "		30	0.075
Three "		30	0.083
Four "		30	0.075
" "		60	0.071

TABLE VII.

*Influence of Excess of Decalcifying Salts on the Concentration of Total Nitrogen and Fibrin in Plasma.*

Blood was drawn mixed with sufficient oxalate to prevent clotting. Salt was then added to portions of blood to concentration indicated. Blood centrifuged and plasma analyzed in usual way.

Sample No.	Concentration of oxalate. <i>per cent</i>	Total N per 100 cc.	Fibrin N per 100 cc.
		gm.	gm.
1	0.1	1.16	0.024
	1.1	1.018	0.029
	2.0	0.958	0.025
2	0.5	1.0	0.049
	1.0	0.929	0.051
	2.0	0.874	0.051
3	0.5	0.898	0.051
	1.0	0.862	0.047
	2.0	0.746	
To plasma from 0.5 per cent oxalated blood (Sample 3) oxalate was added to make 2 and 5 per cent.	2.0	0.887	
	5.0	0.887	

*Influence of the Oxalate Concentration of the Plasma.*—It was found (Table VII) that the amount of oxalate present in the plasma had no effect on the fibrin, but that variation in the concentration of oxalate in the whole blood before centrifugation has a considerable effect on the total nitrogen content of the plasma, presumably because of effects on the water distribution between cells and plasma. Consequently in order to obtain accurately comparable results on different plasmas a constant concentration of oxalate must be used. We add 0.5 gm. of potassium oxalate per 100 cc. of whole blood.

#### SUMMARY.

Methods are described for separation of the fibrin, globulin, and albumin of blood plasma in such a manner that they may be determined by the Kjeldahl method.

#### BIBLIOGRAPHY.

- Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61; 1918, xxxiv, 97.  
Reiss, E., *Beitr. chem. Physiol. u. Path.*, 1903, iv, 150.  
Robertson, T. B., *J. Biol. Chem.*, 1912, xi, 179; also *Physical chemistry of the proteins*, New York, 1918.  
Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 295.



## EXPERIMENTAL STUDIES ON DIABETES.

### SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

#### 1. GROSS ANATOMIC RELATIONS OF THE PANCREAS AND DIABETES.

By FREDERICK M. ALLEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 8, 1919.)

The basis of the present studies has been a form of diabetes produced by removal of the greater part of the pancreas of animals, leaving a remnant about the duct secreting normally into the duodenum, thus avoiding the rapidly fatal cachexia of total pancreatectomy and also the pancreatic sclerosis and deficient digestion of Sandmeyer diabetes and affording a very close and satisfactory reproduction of the clinical disorder.<sup>1</sup> Because of the importance of relations between pancreas mass and body mass, and between the total pancreas and the size of remnant with which diabetes occurs, a summary will first be given of these anatomic facts in dogs, taken from the records of all the experiments suitable for this purpose.

#### *Accuracy of Estimation of the Size of the Pancreas Remnant.*

The method of operation and estimation of the remnant has been previously described.<sup>2</sup> In brief, a piece of the removed tissue is trimmed to imitate the remnant left *in situ*; this piece and the total tissue removed are weighed, and the absolute and relative weights of the remnant and total pancreas are thus reckoned. It is important to combine sight and touch to obtain the greatest accuracy, for feeling the comparative thickness and mass often corrects mistakes of the eye. Though practice is important, the range of error was practically constant throughout this series, and differences depended

<sup>1</sup> All operations were performed under ether anesthesia.

<sup>2</sup> Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, Chapter X.

not so much upon care, skill, or chance as upon differences in the shape of the pancreas. When, as frequently, a piece can be cut from directly beside the remnant, of practically the same shape and consistency, the agreement is generally close; but when a piece of different form and structure must be used, the error is likely to be greater.

As shown in Table I, 68 dogs freshly dead from natural or experimental causes were subjected to the usual operation and the actual remnant was immediately removed and compared with the estimated one. The total of the pancreas weights of the 68 dogs was 1,812.8 gm. The sum total of the estimated remnants was 197.5 gm., of the actual

TABLE I.  
*Accuracy of Estimation of the Size of the Pancreas Remnant.*

Material.	No. of animals.	Combined weight of.			Maximum individual error.		
		Total pancreas.	Estimated remnants.	Actual remnants.	Absolute.	Relative, as fractions of total pancreas.	
						Estimated remnant.	Actual remnant.
Dead dogs.....	68	1,812.8	197.5	196.8	1.1	$\frac{1}{16}$	$\frac{1}{16} - \frac{1}{17}$
" puppies.....	12	74.0	9.1	9.2	0.4	$\frac{1}{8} - \frac{1}{10}$	$\frac{1}{8} - \frac{1}{9}$
Total pancreatectomies.....	18	419.7	45.2	43.4	0.8	$\frac{1}{5}$	$\frac{1}{6}$

remnants 196.8 gm. The greatest individual error occurred when the total pancreas weighed 70.7 gm., the estimated remnant 5.1 gm., and the actual remnant 6.2 gm. Thus, the absolute difference between the remnants was 1.1 gm., and when expressed as usual in the form of fractions, the estimated remnant was approximately  $\frac{1}{16}$  and the actual remnant between  $\frac{1}{16}$  and  $\frac{1}{17}$  of the total pancreas. The absolute error is naturally apt to be greatest when, as here, the dog, pancreas, and remnants are large; but only rarely is the relative error sufficient to change the denominator of the fraction by more than one digit. In this series of 68 dogs, the estimated and actual remnants were equal or within 0.1 gm. of each other in 25 instances. In the other 43 animals, the estimated remnant weighed more than

the actual remnant in 22 cases, and less in 21. The difference between the estimated and actual remnants was 0.2 gm. in 17 instances, 0.3 gm. in 9 instances, 0.4 gm. in 6 instances, 0.5 gm. in 4 instances, 0.6 gm. in 2 instances, 0.7 gm. in 1 instance, 0.8 gm. in 1 instance, 0.9 gm. in 2 instances, and 1.1 gm. in the extreme instance mentioned.

A similar procedure was carried out upon twelve dead puppies, ranging in age from 2 weeks to 10 months. As the animals and organs were smaller, the absolute error was less, but the relative error was about the same. It was greatest in the largest pup, which was 10 months old, and in which the pancreas weighed 13.8 gm., the estimated remnant 1.4 gm., and the actual remnant 1.8 gm. In every other instance the agreement was within 0.1 gm.

The conditions of operation upon living animals were fully reproduced when partial pancreatectomy was performed in the course of total pancreatectomy upon eighteen dogs. The general summary is shown in Table I. The estimated and actual remnants were equal or within 0.1 gm. of each other in 4 instances. They differed by 0.2 gm. in 3 instances, by 0.3 gm. in 3 instances, by 0.4 gm. in 2 instances, by 0.6 gm. in 5 instances, and by 0.8 gm. in 1 instance. This maximum error occurred when the total pancreas weighed 31.4 gm., the estimated remnant 3.9 gm., =  $\frac{1}{8}$ , and the actual remnant 3.1 gm., =  $\frac{1}{7}$  of the total pancreas.

It may be concluded from these figures that the method of estimation is exact enough to be used for statistical studies. But close as is the agreement on the average and in the majority of cases, there is a large minority of errors of a magnitude sufficient to determine the occurrence or absence of diabetes. Accordingly, if the influence of any agencies in causing or preventing diabetes is to be tested with the aid of this operative method, these possible limits of error must be considered in judging the results.

#### *Body Weight, Pancreas Weight, and the Tendency to Diabetes.*

If the operative estimates are accepted as accurate to the degree above shown, a series of dogs subjected to partial pancreatectomy is available for analysis (Table II).

TABLE II.  
*Analysis of Results of Partial Pancreatectomies.*

No. of dogs.	Body weight.	Pancreas weight.			Weight of pancreas per kilo of body weight.			Maximum fraction of pancreas with which diabetes occurred.
		Average.	Maximum.	Minimum.	Average.	Maximum.	Minimum.	
1	2	9.0			4.50			
2	3	9.9	10.0	9.7	3.30	3.33	3.23	
8	4	12.1	16.8	8.9	3.02	4.20	2.22	$\frac{1}{2} - \frac{1}{3}$
18	5	14.2	20.5	8.7	2.84	4.10	1.74	$\frac{1}{3}$
20	6	15.5	18.2	11.2	2.59	3.03	1.87	$\frac{1}{3}$
23	7	17.5	29.4	10.9	2.50	4.20	1.56	$\frac{1}{3}$
26	8	19.7	28.3	12.6	2.46	3.54	1.58	$\frac{1}{3} - \frac{1}{4}$
33	9	22.0	31.1	16.5	2.44	3.45	1.83	$\frac{1}{5}$
35	10	22.3	31.3	14.0	2.23	3.13	1.40	$\frac{1}{3}$
25	11	25.5	38.9	17.3	2.32	3.54	1.57	$\frac{1}{3} - \frac{1}{4}$
36	12	25.7	42.5	15.5	2.14	3.54	1.30	$\frac{1}{3}$
27	13	27.8	39.4	18.7	2.14	3.03	1.44	$\frac{1}{3}$
23	14	30.2	44.1	20.5	2.16	3.15	1.46	$\frac{1}{3}$
34	15	31.4	50.3	20.6	2.09	3.35	1.37	$\frac{1}{3}$
22	16	35.9	63.4	27.3	2.24	3.96	1.71	$\frac{1}{3}$
13	17	33.7	50.3	26.6	1.98	2.96	1.56	$\frac{1}{3} - \frac{1}{4}$
27	18	36.5	49.6	23.1	2.03	2.76	1.28	$\frac{1}{3}$
13	19	34.8	52.9	28.1	1.83	2.78	1.48	$\frac{1}{3}$
11	20	35.5	46.1	23.5	1.77	2.30	1.17	$\frac{1}{3}$
7	21	41.5	54.5	29.1	1.98	2.60	1.39	
2	22	35.1	38.5	31.8	1.59	1.75	1.45	$\frac{1}{3} - \frac{1}{4}$
6	23	46.5	54.7	38.3	2.02	2.38	1.66	$\frac{1}{3}$
5	24	45.0	51.9	39.4	1.87	2.16	1.64	$\frac{1}{3}$
2	25	66.3	94.4	38.0	2.65	3.77	1.52	
7	26	54.2	67.5	43.5	2.08	2.60	1.67	$\frac{1}{3} - \frac{1}{10}$
1	27	51.1			1.89			
1	28	40.8			1.46			
2	29	73.8	95.3	52.0	2.54	3.28	1.79	
1	30	35.9			1.19			
1	31	70.7			2.28			
2	34	72.8	88.9	56.8	2.14	2.62	1.67	
1	36	58.9			1.63			
1	37	82.8			2.24			
1	42	81.8			1.95			

The weight of the pancreas ranged from a minimum of 1.17 gm. to a maximum of 4.5 gm. per kilo of body weight. These figures are derived from animals which were chosen as apparently normal in all

respects, free from any disturbing factors such as notable obesity or emaciation, extreme age or youth, pancreatic or other disease, etc., and in which, moreover, the normality of the pancreatic tissue was in a high proportion of instances confirmed by microscopic examination. They are therefore of statistical value from the pure anatomic standpoint, and also as a basis of comparison for some of the ensuing studies.

A detailed record was kept of the sex, apparent age, breed, color and character of coat, and other characteristics of all animals. Analysis indicates (*a*) that sex is immaterial, and also (*b*) that in adult animals variations of age are without perceptible influence in regard to the pancreas weight. (*c*) No breeds were found with characteristically large or small pancreas. (*d*) On the other hand, the numerous crosses and admixtures of different breeds were suggestive. Any one handling large numbers of dogs will observe that while characters are generally blended, the size and form of organs are among those which are sometimes transmitted almost like Mendelian units. Thus, the large square bulldog head with its undershot jaw may be found on a fox-terrier body, and examples of obvious bastardy in legs, tail, coat, and other features are too common to mention. The uncertain ancestry of all the animals and the mixture in the majority make tabulation or exact conclusions impossible for both (*c*) and (*d*). But there is no doubt that the variations in size due to cross-breeding pertain also to visceral organs. One of the clearest examples is the occasional finding of the deep thorax and large lungs of a greyhound in a mongrel with the other characters of some other breed or mixture. The impression was gained that in the crossing of large and small dogs, the size of the pancreas in the offspring is usually a blend roughly proportioned to the body weight, but in some instances it is chiefly derived from either the large or the small parent, so that the ratio of pancreas weight to body weight is altered, and the animal has an exceptionally large or small pancreas in proportion to the size of its body.

Dogs offer unique advantages for tracing a range of relations between body weight and pancreas weight. No other mammal exhibits such a gamut of sizes as here represented between the 2 and 42 kilo weights for normal adults of the same species. In a broad and

general way, with allowance for the exceptions mentioned in the preceding paragraph, the table indicates a lowering of the proportional size of the pancreas as the body weight rises. The rule in this general sense applies to all three columns, of average, maximum, and minimum weights. In other words, small dogs generally possess more pancreas tissue in proportion to their body mass than large dogs. According to well known laws, the basal metabolism of small dogs per kilo is higher than that of large dogs, and with allowances for activity, warmth of coat, and other modifying factors, their actual daily metabolism in proportion to weight is doubtless higher. As one of the principal studies of the entire investigation pertained to the relation of the pancreatic function respectively to the body mass and to the metabolism, it was interesting to observe that dogs seem normally to be provided with pancreatic tissue rather in proportion to their metabolism than to their body mass.

The observations were also directed to determine whether a uniform mathematical law can be established for the occurrence of diabetes whenever a fixed proportion of the pancreas is removed, or whether general or individual exceptions exist. With regard to the statements in the preceding paragraph, it might be inquired whether small dogs have a larger endowment of pancreas corresponding to larger needs, or merely a greater margin of safety. It is known from veterinary literature that dogs are occasionally subject to spontaneous diabetes. In human pathology, a tendency to diabetes has sometimes been attributed either to small size of the pancreas in gross, or to deficiency of islands. In view of the impracticability of any extensive counting of islands, there was a question whether dogs having a small pancreas would show a corresponding susceptibility to diabetes, or whether differences in the functional capacity of equal masses of pancreatic tissue would be demonstrable, due to variations in island content or other differences. Dogs exhibit contrasts of nervous and phlegmatic temperaments almost as extreme as those of men. Also some varieties of dogs have resulted from close inbreeding, and special characters have sometimes been cultivated at the price of constitutional vigor. As shown in Table II, wide differences were observed in the size of the pancreas remnant with which diabetes occurred; but these were rare and did

not conform to any of the causes above considered. Special attention was paid to the few instances in which diabetes occurred with  $\frac{1}{2}$  to  $\frac{1}{4}$  of the pancreas present, but only after several years was it demonstrated that the explanation lay in inflammatory changes, as described in a paper to be published later. For the most part, large dogs develop diabetes more readily and are more satisfactory for the purpose than small ones, but accessory factors enter in. The small pancreas remnant in a small dog is traumatized in operation to a relatively greater extent than the larger remnant of a large dog. This difference is overbalanced by the greater vigor and resistance of large dogs. The small ones are more subject to loss of appetite, cachexia, and distemper, which interfere with the development of active diabetes. Owing to the modifying influences, no uniform rule can be deduced from the last column of Table II. The present figures, covering a larger number of animals, confirm previous findings<sup>2</sup> that, with few exceptions, mild diabetes occurs when the remnant is  $\frac{1}{2}$  to  $\frac{1}{4}$  of the pancreas, and severe diabetes when the remnant is about  $\frac{1}{16}$  of the pancreas. The internal secretory capacity of equivalent fractions of pancreatic tissue is uniform as far as these observations could determine, with no sign of variations due to gross size of the organs, differing island content, nervous control, or constitutional vigor or degeneracy. Except for inflammatory changes as mentioned, no special tendency to diabetes was evident in any dog of the series.

The observations outline the limits of error under this procedure. A demonstration of the influence of any agency in producing or preventing diabetes, by a comparison of test animals and controls, must rest upon uniform results in a considerable series, and the differences in question must generally amount to several grams of pancreas tissue. Other experiments have shown that a partially depancreatized animal, which has merely a lowered tolerance and cannot be made diabetic by any quantity or duration of feeding, may be so close to the verge that diabetes results from the removal of only a fraction of a gram of additional pancreatic tissue. Also definite variations in the severity of diabetes may be produced, for

example by overfeeding. The most delicate method, therefore, consists in tests upon the same rather than upon different animals. Due attention must always be paid to accidental modifying factors, especially body weight, pancreatitis, and cachexia.

### *Hypertrophy of the Pancreas Remnant.*

The former series of experiments contained several examples of great hypertrophy of the pancreas remnant. Thus, in Dog 104<sup>3</sup> the pancreatic tissue left at the original operation on Oct. 1 was estimated at less than 6 gm. On Nov. 27, 6 gm. of tissue were removed, and the remnant found at autopsy on Dec. 23 weighed 8.1 gm. Also<sup>4</sup> in Dog 151 the remnant estimated at operation on Nov. 21 was 3.2 gm., while that found at autopsy on Dec. 4 was 11.3 gm.; and in Dog 152 the remnant estimated at operation on Nov. 24 was 4.4 gm., while that found at operation on Dec. 5 was 10.4 gm. In Dog 125<sup>5</sup> the remnant estimated at operation on Oct. 26 was 2.4 gm., and that found at autopsy on Nov. 8 was 7.7 gm. In Dog 148<sup>6</sup> the remnant estimated at operation on Nov. 16 was 3.3 gm., and that found at autopsy on Dec. 19 was 13.3 gm.

Hypertrophy to such an extreme degree is highly exceptional. Tables were compiled from a series of dogs which had undergone removal of most of the pancreas in single operations and were free from any known disturbing influence. The data concerning alterations in weight of the pancreas remnant are summarized in Table III.

If  $\pm 0.4$  gm. is accepted as a rough estimate of the possible limits of error, it is seen that in 35 instances the weight of the remnant remained practically stationary. The 17 cases of atrophy represented essentially traumatic fibrosis. Hypertrophy occurred in the majority of cases in the total series, but was generally slight, so that in only 12 instances was the remnant found more than doubled in size. To save reproduction of extensive detailed data, the following points may be noted as developed by analysis.

*Occurrence.*—Changes occur in the size of the pancreas remnant beyond any possible error of estimation. In calculations of the proportion of pancreas removed, the operative estimate should be used in preference to the weight of tissue at autopsy.

<sup>3</sup> Allen,<sup>2</sup> pp. 485-486.

<sup>4</sup> Allen,<sup>2</sup> p. 490.

<sup>5</sup> Allen,<sup>2</sup> p. 491.

<sup>6</sup> Allen,<sup>3</sup> p. 959.

*State of Nutrition.*—Precautions were taken to make sure that the changes in weight did not represent mere differences in fullness and emptiness of the acini. The series also included changes of the nutritive state in both directions. Gain of body weight did not necessarily correspond to increase in the pancreas remnant. Also, though the general wasting in emaciation affects the pancreas, the hypertrophy was all the more evident because some of the most marked examples were found in animals which lost a large proportion of their body weight between operation and autopsy.

*Character of Tissue.*—In certain instances, especially soon after operation, the increase of weight may be due to inflammatory tissue,

TABLE III.

*Alterations in the Weight of the Pancreas Remnant after Partial Pancreatectomy.*

No. of instances in which weight of pancreas remnant at autopsy was found within ± 0.4 gm. of estimate at operation .....	35
No. of instances of atrophy beyond the above limit of error.....	17
Average atrophy of the group (in percentage of estimated weight of remnant).....	27.4 per cent.
Maximum atrophy .....	45 " "
No. of instances of hypertrophy to less than 150 per cent of the estimated weight... 28	
" " " " 150 per cent of the estimated weight.....	30
" " " " 200 " " " " " .....	30
" " " " 300 " " " " " .....	8
" " " " 400 " " " " " .....	4

but this is not the true hypertrophy referred to. Sometimes also fairly normal parenchyma may be distorted or more or less encapsulated by superficial scar tissue. But in the best examples the remnant is free from perceptible sclerosis and consists of lobulated parenchyma normal in appearance and consistency. Microscopic examination confirms the absence of fibrosis. The acini may be particularly large and crammed with secretion. As described for Dogs 148 and 151<sup>7</sup> islands may be scanty as if the hyperplasia had been limited chiefly to the acinar tissue, or fairly abundant, as if the islands had kept pace with the proliferation of the other structures.

<sup>7</sup> Allen,<sup>2</sup> pp. 761, 969.

*Size of Remnants.*—The absolute size of the remnant is not a determining factor in hypertrophy. The remnants in Dogs 104, 125, 148, 151, and 152, mentioned above, were of various sizes. The later series includes remnants of 8 to 12 gm. which increased to 14 to 22 gm., also a remnant of 0.8 gm. which increased to 2 gm., one of 1.6 gm. which increased to 5.4 gm., and one of 1.8 gm. which increased to 6.5 gm. Correspondingly, the relative size of the remnants did not govern hypertrophy. Some of the larger remnants referred to were as much as  $\frac{1}{4}$  of the pancreas, while the 0.8 gm. remnant mentioned was only  $\frac{1}{16}$  of the total organ.

*Relation to Diabetes.*—It follows from the last statement that diabetes was not a determining factor in hypertrophy, which was present or absent in diabetic and non-diabetic animals indiscriminately. In other words, the stimulus did not apparently originate from lack of either the internal or external function of the pancreas. A still more important question is to what extent the hypertrophy was able to prevent or cure diabetes. In some instances the new formed tissue appeared functionally equivalent to the old, for not only was diabetes checked, but also to reproduce it a further resection of tissue was necessary to reduce the remnant to the size requisite in original operations. On the other hand, diabetes sometimes stopped when there was little or no hypertrophy of the remnant; also hypertrophy was frequent with continuance of diabetes. Closer study of these results, which appear contradictory at first glance, brings out the following explanatory facts. (a) The traumatic inflammation following operation is generally a factor in producing diabetes in cases with fairly large remnants. When by any means symptoms are prevented until the inflammation has subsided, it is sometimes found that the animal is no longer diabetic even though there has been little or no increase in the size of the remnant. (b) Inflammation may injure chiefly the islands, to such an extent that the animal remains diabetic after the inflammation has subsided notwithstanding hypertrophy of the remnant. If a functional injury may be assumed, as seems to be true in human diabetes, an explanation is afforded of the existence of diabetes even when the hypertrophic remnant contains numerous islands, as in Dog 151,<sup>6</sup> but this point is still doubtful. (c) Overfeeding furnishes the most

frequent explanation, and may be put to valuable use. For example, severe acidosis is obtainable only when the dogs can digest large quantities of fat, and dogs with small pancreas remnants can seldom do this. But by cautious overfeeding with carbohydrate after operation, mild diabetes may be kept up, while in a certain proportion of animals marked hypertrophy of the remnant is taking place. Islands are thus injured or destroyed, while the great increase of acinar tissue enables a satisfactory digestion of fat.

*Age of Animals.*—The animals mentioned above were fairly young, mostly 2 or 3 years of age. But both hypertrophy and its absence have been noted in dogs at all ages. Dog 146<sup>7</sup> was senile, yet the pancreas remnant in 3 weeks increased from 2.8 gm. to 6.3 gm. Also the records of puppies in a subsequent paper indicate no remarkable regenerative activity, just as the damaged pancreas of diabetic children fails to recover to any greater extent than that of adults.

*Time Element.*—It is impossible to determine by weight the beginning of hypertrophy, because the operation ordinarily is followed by inflammatory exudate and infiltration. Epithelial hyperplasia begins almost simultaneously and sometimes proceeds with astonishing rapidity. The most rapid increase observed was in Dog 152; namely, the growth of an estimated 4.4 gm. remnant to 10.4 gm. in 11 days. The hypertrophy seems to be mostly complete within the first few weeks after operation. The result is permanent, as shown in observations covering 3 years and over.

*Cause of Hypertrophy.*—The occasional hypertrophy of a mass of pancreatic tissue to several times its size, in contrast to the relatively slight increase in the great majority of instances, seemed a phenomenon worthy of attention for possible application in the treatment of human diabetes. Various attempts, by trimming remnants in different shapes, by multiple incisions through the surface, and by leaving protruding ducts as possible origins of proliferation, gave no consistent results. Here, as in certain of the preceding paragraphs, it is necessary to anticipate some of the findings of the microscopic study. The recently traumatized tissue shows a mass of inflammatory, degenerative, and regenerative changes, and it is inferred that the predominance of any of the three determines the result.

There is no warrant for attempting to stimulate regeneration of the human pancreas by mere trauma. Some more hopeful method may ultimately be worked out. The observations are important as confirmation of other evidence of the regenerative power of the pancreas in postembryonic life.

*Repeated Operations.*

In Table III, it was possible only to pick out the maximum weight of the pancreas remnant with which different animals developed diabetes in single operations. Each animal in Table IV was subjected to successive removals of pancreatic tissue, so that the exact reduction of the organ requisite for diabetes was sometimes determined within a fraction of a gram. The results in general confirm those of Table III. The traumatic inflammation of repeated operations accounts for the occasional occurrence of diabetes with large remnants. The degree of hypertrophy, obtained as a total by adding together the weights of tissue removed in operations and present at autopsy, was similar to that observed with single operations.

The observations were mostly long, generally continuing for months or years after the last operation. The operations were from two to five in number, and the intervals between them ranged from 2 weeks to 32 months. The time elapsed made no demonstrable difference in the susceptibility to diabetes. This conclusion has reference to the suggestion of some former writers that animals may develop "immunity" to the lack of pancreatic tissue, particularly by vicarious action of other glands. Such ideas are probably explained by the fact that these workers dealt with atrophic pancreas remnants lacking duct communication with the bowel, so that (a) in subsequent operations some fragments were missed, and (b) cachexia hindered the development of typical diabetes.

The same observation supports the conclusion of papers to follow that partially depancreatized animals show no inherent increase of tendency to diabetes with time.

TABLE IV.  
*Repeated Operations.*

Dog No.	Normal body weight.	Total pancreas weight.	Remnant.		Hyper trophy.	Time elapsed between first and last operations.	Remarks.
			Estimated weight.	Frac- tion.			
B2-02	10.5	30.5	6.8	$\frac{1}{2} - \frac{1}{3}$	gm.	32	Very mild diabetes, due to traumatic inflammation.
B2-05	6.5	11.5	1.2	$\frac{1}{3} - \frac{1}{4}$		$\frac{1}{2}$	Mild diabetes, stopped by distemper.
B2-43	11.1	18.8	5.1	$\frac{1}{4}$	5.1-5.8	$\frac{1}{2}$	Slow onset of mild diabetes.
B2-51	9.75	26.2	2.75	$\frac{1}{6} - \frac{1}{10}$		1	Severe diabetes.
B2-53	10.25	24.5	3.5	$\frac{1}{4}$	3.5-4.5	$\frac{1}{2}$	Cachexia.
B2-63	24.4	49.0	7.4	$\frac{1}{3} - \frac{1}{4}$	7.4-14.2	22	Diabetes resulted from subsequent operations removing 1.32, 0.36, and 0.22 gm.
B2-88	14.4	30.7	2.8	$\frac{1}{11}$	2.75-2.75	1 $\frac{1}{2}$	Subcutaneous graft also present. Diabetes followed its removal.
B2-89	13.1	30.1	2.7	$\frac{1}{11}$		11	Diabetes checked by emaciation; restored by removal of additional 0.65 gm.
C3-20	24.0	51.9	11.5	$\frac{1}{2} - \frac{1}{3}$	11.5-20.2	4 $\frac{1}{2}$	Mild diabetes after four subsequent operations removing total of 5.3 gm. Remnant at autopsy weighed 14.9 gm.
C3-27	16.25	38.4	4.4	$\frac{1}{8}$	4.4-8.7	8	Severe diabetes after removal of additional 0.9 gm.
C3-45	10.8	27.5	3.7	$\frac{1}{7} - \frac{1}{8}$		4 $\frac{1}{2}$	Transitory diabetes; permanent after removal of additional 0.3 gm.
C3-58	15.9	43.8	2.9	$\frac{1}{18}$	2.9-4.25	4 $\frac{1}{2}$	Diabetes controlled by diet. Dog emaciated to 9 kilos.
C3-86	15.0	35.9	2.9	$\frac{1}{18} - \frac{1}{19}$	2.9-1.8	7 $\frac{1}{2}$	Anorexia prevented diabetes till additional 0.2 gm. was removed. Sclerosis and atrophy of remnant.
C3-98	13.4	27.9	2.1	$\frac{1}{19}$	2.1-4.4	8	Diabetes controlled by diet. Later two operations removing total of 1.45 gm. necessary to bring back diabetes.
D4-63	15.25- 18.7	46.5	4.6*	$\frac{1}{10}$		7 $\frac{1}{2}$	Mild diabetes.
D4-85	10.0	25.9	2.0	$\frac{1}{18}$		1	Severe "
D4-86	15.9	35.5	3.9	$\frac{1}{6}$	3.9-10.8	1 $\frac{1}{2}$	" "
D4-90	36.9	82.8	1.9	$\frac{1}{18} - \frac{1}{19}$		1	" "
D4-96	11.6	24.2	1.8	$\frac{1}{18} - \frac{1}{19}$	1.8-2.4	3 $\frac{1}{2}$	" "
E5-00	15.25	35.0	3.5	$\frac{1}{10}$		$\frac{1}{2}$	" "
E5-16	18.0	34.0	6.75	$\frac{1}{2}$	6.75-9.0	$\frac{1}{2}$	Mild "

\*Weight at autopsy.

*Pancreas Weight in Relation to Reduced Body Weight.*

The filling of the acini with zymogen during short fasts and the discharge on functional stimulation are probably sufficient to cause considerable changes in the gross weight. On the other hand, long fasting is accompanied by shrinking of the acinar cells and diminution

TABLE V.  
*Dogs with Malnutrition.*

Dog No.	Normal body weight.	Final body weight.	Pan- creas weight.	Weight of pan- creas per kilo of normal body weight.	Weight of pan- creas per kilo of final body weight.	Remarks.
	kg.	kg.	gm.	gm.	gm.	
C3-17	12.6	10.5	22.2	1.76	2.11	Poor appetite for 3 wks. 110 gm. of glucose subcutaneously 3 hrs. before death.
D4-27	12.5	8.8	22.1	1.77	2.51	Overfatty diet for 3 mos. Fat intoxication. 40 gm. of glucose subcutaneously 6 hrs. before death.
D4-31	10.6	8.0	12.1	1.14	1.51	Fasting for 2 wks., followed by overfatty diet for 6 wks. Fat intoxication.
F6-54		5.6	24.9		4.45	Thin when received. Killed with phosphoric acid intravenously.
F6-95	4.3	3.7	8.5	1.98	2.30	Fasting for 12 days; poor appetite following 12 days. Death from acetoacetic acid intravenously.
G7-21		15.0	30.6		2.04	Very thin when received.
G7-87		6.4	14.7		2.30	" " " " Killed with bicarbonate intravenously.
G7-89	18.0	14.0	47.8	2.66	3.41	Fasting and phlorizin 1 wk.; appetite poor 1 wk.; then killed with acid and alkali intravenously.
G7-99		8.0	15.0		1.87	Thin when received. Death from shock and bicarbonate.
C3-01		14.9	29.6		1.99	Collie; very thin when received.

of zymogen, so that in some cases the pancreas consists entirely of involuted rounded cells, with only rare traces of zymogen and little or no visible acinar arrangement.<sup>8</sup> The gross organ is softer and smoother, less lobulated, sometimes almost translucent or gelatinous

<sup>8</sup> Allen,<sup>2</sup> Chapter XXI, also Figs. 2, 3, and 4.

in areas, and obviously below normal weight. The present series did not include extremely short or long fasts, but only such intermediate degrees of abstinence or reduced diet as caused appreciable reduction of body weight.

Table V is made up of dogs which were either received in a very emaciated condition or suffered more or less loss of weight under

TABLE VI.

*Fasting Dogs.*

Dog No.	Normal body weight.	Final body weight.	Pancreas weight.	Weight of pancreas per kilo of normal body weight.	Weight of pancreas per kilo of final body weight.	Remarks.
	kg.	kg.	gm.	gm.	gm.	
C3-15	14.5		17.3	1.19		Long voluntary fast. 150 gm. of glucose by stomach 1½ hrs. before death.
D4-01	14.5	10.6	22.8	1.57	2.15	Undernutrition for 54 days, followed by fasting for 4 days.
F6-50	6.0	4.2	16.7	2.78	3.98	Fasting for 11 days with occasional doses of hydrochloric acid.
F6-52	6.3	5.0	13.4	2.13	2.68	Fasting for 8 days. Killed with acid sodium phosphate intravenously.
F6-79	10.2		23.1	2.26		Fasting for 20 days with large doses of hydrochloric acid.
F6-99	6.4	4.5	12.2	1.91	2.71	Fasting for 17 days. Death from 20 gm. of lactose intravenously.
G7-00	5.5	3.5	13.6	2.47	3.89	Fasting for 25 days. 25 gm. of glucose intravenously on last day. Milk vomited.
G7-01	5.5	4.4	10.3	1.87	2.34	Fasting for 18 days. Death from dextrin intravenously.
B2-52	12.8	9.9	23.5	1.84	2.38	Fasting for 16 days before operation.
B2-57	11.1	8.6	21.6	1.95	2.51	" " 18 " " "
B2-58	11.8	9.1	18.0	1.53	1.98	" " 18 " " "

observation. The animals of Table VI underwent actual fasting for various periods, sometimes together with other experimental procedures as noted. Those of Table VII received phlorizin as stated, subcutaneously, besides the fasting or undernutrition imposed.

In a majority of instances, both the absolute and relative weights of the pancreas fall within the normal extremes shown in Table II,

TABLE VII.  
*Fasting or Undernutrition with Phlorizin.*

Dog No.	Normal body weight.	Final body weight.	Pan- creas weight.	Weight of pan- creas per kilo of normal body weight.	Weight of pan- creas per kilo of final body weight.	Remarks.
	kg.	kg.	gm.	gm.	gm.	
C3-54	19.5	15.0	20.5	1.05	1.37	Fasting with 1 gm. of phlorizin daily for 10 days. Lard feeding and bicarbonate intravenously on day of death.
C3-57	14.5		13.9	0.96		Fasting for 9 days; pure fat feeding on last 3 days. 1 gm. of phlorizin daily.
C3-69	17.5	13.9	23.0	1.31	1.65	Undernutrition, followed by fasting for 5 days with 1 gm. of phlorizin daily. Glucose intravenously on day of death.
C3-99	22.5	17.0	31.2	1.39	1.83	Protein-fat diet with 0.5 or 1 gm. of phlorizin daily for 50 days.
D4-00	13.2	10.8	17.9	1.36	1.66	Protein-fat diet with 0.5-0.75 gm. of phlorizin every day or two for 50 days. Glucose and bicarbonate on day of death.
E5-07	15.0	11.4	30.8	2.05	2.70	Fasting for 10 days with four doses of 1 gm. of phlorizin. 50 gm. of glucose by stomach 2 days before death.
E5-33	17.5	13.9	35.0	2.00	2.52	Fasting for 1 wk. with five doses of 0.5 gm. of phlorizin.
F6-22	13.6	9.8	31.7	2.33	3.24	Fasting for 10 days with 0.5 gm. of phlorizin daily. 50 gm. of glucose subcutaneously on day before death.
F6-25	12.0	7.1	18.5	1.54	2.63	Fasting for 11 days with three doses of 0.5 gm. of phlorizin.
F6-32	17.6		27.0	1.53		Fasting for 6 days with three doses of 1 gm. of phlorizin. Bicarbonate on last 2 days.
F6-33	14.2		22.7	1.60		Fasting for 9 days. Bicarbonate on 2 days before death, and sodium chloride on 2 days before that.
F6-67	16.0	11.0	31.1	1.94	2.83	Fasting for 16 days with seven doses of 1 gm. of phlorizin.
F6-91	12.7	9.0	20.3	1.60	2.26	Partial nephrectomy. Fasting for 15 days with four doses of 1 gm. of phlorizin. Fed meat on 11th day of fast. 25 gm. of glucose subcutaneously on 13th day.

TABLE VII—*Concluded.*

Dog No.	Normal body weight. kg.	Final body weight. kg.	Pancreas weight. gm.	Weight of pancreas per kilo of normal body weight. gm.	Weight of pancreas per kilo of final body weight. gm.	Remarks.
G7-28	4.5		10.9	2.42		Fasting for 5 days with three doses of 0.5 gm. of phlorizin. Butyric and aceto-acetic acids intravenously.
G7-75	12.6	11.5	18.5	1.47	1.61	Fasting for 5 days with two doses of 1 gm. of phlorizin.
G7-76	11.2	9.0	20.4	1.82	2.26	Fasting for 8 days with four doses of 1 gm. of phlorizin. Early pregnancy and abortion.
G7-82	15.0		24.8	1.65		Fasting for 7 days with three doses of 1 gm. of phlorizin.
G7-93		11.4	23.7		2.08	Fasting for 4 days with one dose of 0.5 gm. of phlorizin on 1st day.
G7-96	11.0	10.0	25.5	2.32	2.55	Fasting for 5 days with two doses of 0.5 gm. of phlorizin.

whether calculated on the normal or the reduced body weight. Also in a majority of instances, they are closer to the average for the reduced than for the normal body weight. The rule has such marked exceptions in both directions that a table of averages could not properly be used. The degree of undernutrition is as great as any employed for controlling even the severest diabetes, and the results do not warrant a conclusion that the therapeutic effects of undernutrition are explainable by an increase of the ratio of pancreas mass to body mass. The islands perhaps shrink less than the acinar tissue in fasting, but there is no basis for a conclusion concerning mass relations.

Any marked changes in the mass of the pancreas due to accompanying experiments would also be revealed by such comparisons. For example, the exceptionally large pancreas weights of Dogs F6-54 and G7-89 in Table V represent the edema of the pancreas sometimes produced by acid injections. The other experimental procedures, such as glucose injections and especially phlorizin poisoning, did not demonstrably alter the gross weight of the pancreas.



## EXPERIMENTAL STUDIES ON DIABETES.

### SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

#### 2. EFFECTS OF CARBOHYDRATE DIETS.

By FREDERICK M. ALLEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 8, 1919.)

The belief underlying the dietetic treatment of diabetes has been that the disorder consists in the weakness of a bodily function, which is broken down by overstrain and spared or strengthened by rest. A coexistent and equally firmly rooted belief has been that some or perhaps most human cases are inherently progressive, on account of continuing toxic, infectious, or undetermined factors, so that they become worse and lead to death after a longer or shorter time in spite of dietary restrictions. Attention was previously directed<sup>1</sup> to the importance of determining this point positively in dogs, which are free from constitutional tendencies. This fundamental question of the possibilities and limitations of injuring assimilation and shortening life by overfeeding and improving tolerance and lengthening life by restricted diet was not fully settled by the experiments of Thiroloix and Jacob<sup>2</sup> or the present writer, and papers to follow will be devoted particularly to this subject. Microscopic changes will be mentioned briefly here and treated more completely later.

#### *Effect of Starch-Rich Diet.*

Dog B2-65.—Female; mongrel; black, slightly shaggy; age 5 years; rather thin; weight 17.5 kilos. May 25, 1914. Removal of pancreatic tissue weighing 28.9 gm.<sup>3</sup> Remnant about main duct estimated at 3.4 gm. ( $\frac{1}{3} - \frac{1}{4}$ ). The urine

<sup>1</sup> Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, Chapter X.

<sup>2</sup> Thiroloix, J., and Jacob, *Bull. et mém. Soc. méd. hôp. Paris*, 1910, xxix, series 3, 492; xxx, series 3, 29, 656.

<sup>3</sup> All operations were performed under ether anesthesia.

remained sugar-free on fasting till May 29. Bread and soup diet was then begun, and glycosuria continued absent till June 2, after which date it was continuous. Bits of pancreatic tissue were removed by operations on June 15 and July 9 without apparently altering the steady downward progress. Glycosuria increased; at the outset it was as low as 1.5 per cent in 200 cc. of urine for 24 hours, at the end as high as 9.1 per cent in 2,415 cc. of urine. From July 23 to 28, the sugar was only once below 14 per cent, being thus exceptionally high in percentage. The animal remained in good spirits, but the weight diminished to 9 kilos, with corresponding loss of strength. Death occurred from weakness on July 29.

*Autopsy.*—Except for the emaciation, the autopsy was entirely negative. The pancreas remnant weighed 3.35 gm. The samples of pancreatic tissue for June 15, July 9, and autopsy showed normal acini and successive stages of hydropic degeneration of islands, involving finally a marked reduction in number and size of islands but by no means complete disappearance.

This record may be considered typical of the rapidly fatal course of this form of experimental diabetes on starch-rich diet, corresponding to the rapid emaciation and death in the severest human cases when entirely untreated, though the process is generally more rapid in dogs than in patients.

#### *Breaking Down Tolerance with Glucose.*

*Dog B2-31.*—Female; bull-terrier; white; age 2 years; good condition; weight 11 kilos. Dec. 23, 1913. Removal of pancreatic tissue weighing 19 gm. Remnant about main duct estimated at 2.6 gm. ( $\frac{1}{3}$ – $\frac{1}{2}$ ). Subsequently, bread feeding caused only a fraction of a per cent of glycosuria, which ceased and remained absent even on addition of glucose, until the quantity of glucose reached 200 gm. daily. Glycosuria ranging from 2 to 5.5 per cent in the mixed 24 hour urine of each day continued on the diet of bread and soup with glucose as stated till Feb. 12, 1914. The diet was then changed to meat *ad libitum*. Glycosuria steadily diminished during the succeeding days, till on Feb. 20 it was only 0.22 per cent and was evidently on the point of disappearing. The bread and glucose diet was repeated, with heavier glycosuria than before (up to 5.8 per cent in 24 hour urine of 2,470 cc.), until Feb. 26. The diet of meat *ad libitum* was then resumed, with the result that glycosuria fell as low as 0.9 per cent in 367 cc. of urine on Feb. 28, but rose to 6 per cent in 1,090 cc. of urine on Mar. 6. The condition thereafter was severe diabetes, and the animal was used for therapeutic tests, stringent regulation of diet and weight being necessary to keep glycosuria absent.

This animal was specially suited for the purpose because of an unusually voracious and unfailing appetite and a natural fondness for sweets. Most dogs will not eat so much bread and sugar for so long

a time. Animals and patients alike can evidently bring on diabetes by gluttony, particularly in starch and sugar, when a certain degree of predisposition exists.

Indigestion and diarrhea check the carbohydrate excess and prevent diabetes in many animals, and strong digestive power facilitates the production of experimental and doubtless also of clinical diabetes. Talcum powder mixed with the food, in quantities up to 100 gm. or more daily, has been the chief means used to control diarrhea. The innocuousness of talcum in any amount or duration for such experiments suggested its introduction later as a flour for making pancakes for some patients on extremely low diets.

The downward progress of human diabetes can be further imitated in that the disease in dogs can be made slow or rapid as desired by varying the kind and quantity of food. Like human patients these dogs never regain a tolerance for food equal to that which they possessed before the damage from overfeeding occurred.

The plan of breaking down tolerance with the largest feasible quantities of starch and glucose immediately after operation is of practical importance for securing diabetic dogs with the largest possible pancreas remnants, and hence with the best possible digestive power and vigor. Knowledge of animals and skill in managing their diets are important for success in producing the most valuable test objects for diabetic research; namely, dogs which eat and digest well and appear normal in all respects except for the desired degree of diabetes. The best results require that the investigator should give personal attention to this work.

*Dog B2-80.*—Female; mongrel; black and white; age 3 years; very good condition; weight 17 kilos. Nov. 17, 1914. Removal of pancreatic tissue weighing 25.7 gm. Remnant communicating with both ducts estimated at 7.1 gm. ( $\frac{1}{3}$ - $\frac{1}{4}$ ). The dog was specially valuable, because with this large pancreas remnant immediately after operation she had a tendency to slight glycosuria on plain bread and soup diet, and was easily kept glycosuric by the addition of small quantities of glucose. After a period of sugar freedom on meat diet, the dog was loaned to another laboratory, and was returned with the statement that she could not be made diabetic. Trial with increasing dosage of glucose showed that glycosuria actually remained absent with such quantities as could voluntarily be eaten, and there was danger here as in many cases with large remnants that spontaneous recovery of assimilation due to the delay might spoil the opportunity for diabetes.

Accordingly, beginning Apr. 24, 1915, 500 gm. of glucose were given daily. Diarrhea was controlled by the use of bone-meal and talcum powder, and any portions of the sweetened bread and soup mixture left uneaten were made into firm balls and fed forcibly. Only a 5 day period of this excessive dosage was necessary before it became possible to reduce the quantities with continuance of glycosuria. The animal was later overfed with protein and fat so as to bring on severe diabetes and acidosis. Aug. 14. Died in coma.

*Autopsy.*—The dog possessed abundant fat at death, like some human patients dying of acidosis. The body weight was 17.25 kilos, the weight of the pancreas remnant 13.5 gm. Its tissue was soft, normally lobulated and free from fibrosis, but microscopically consisted of unbroken expanses of acini. Rare small clumps, apparently of alpha cells, were the only remains of islands. Many small ducts showed appearances like vacuolation.

#### *Unsuccessful Attempts to Break Down Tolerance.*

*Dog B2-02.*—Female; Boston terrier; brindle; age 1½ years; weight 10.5 kilos. This dog was received Oct. 25, 1913, and was used for various experiments, especially the removal of successive portions of pancreas tissue, until an operation on Dec. 10, 1914 at length produced diabetes. Glycosuria was present up to Dec. 23 on plain bread and soup feeding. It could readily be produced thereafter by the addition of glucose. But the dog was exceptionally dainty in appetite, would eat only limited quantities of bread, had a strong distaste for sugar, and vomited readily and persistently if forcibly fed. The potential diabetes continued, as proved by the easy production of glycosuria by occasional glucose feedings and the low tolerance for glucose subcutaneously, but active symptoms could never be maintained. Aug. 31, 1915. A bit of pancreas tissue was removed for examination. The condition thereafter was unchanged, namely absence of glycosuria with bread diet, also with the addition of 100 gm. of glucose, but glycosuria with the addition of 200 gm. of glucose; and all such attempts were terminated within a few days by refusal and vomiting. On mixed diet of bread and meat the dog continued in full health at the original weight of 10.5 kilos, until death from rabies, Sept. 19, 1916.

Of numerous animals in which the determining influence of appetite upon the occurrence of diabetes was observed, this one afforded the most striking antithesis to Dog B2-31 above mentioned. The actual assimilative power was much lower, in consequence of operation, in this dog than in Dog B2-31, and the eating habits were alone responsible for the opposite results.

*Autopsy.*—The pancreas remnant weighed 6.8 gm. Grossly it was normal in appearance and consistency, and microscopically the acini were fully normal; but occasional small fibrous trabeculae and a general scarcity of islands indicated a previous inflammation as the probable cause of the potential diabetes with such a large remnant of pancreatic tissue. Also the island cells, both on Aug. 31, 1915, and at autopsy, showed numerous suggestions of vacuolation, generally so slight

as to be doubtful. These anatomic findings are of importance in connection with the question discussed below of the probable ultimate outcome of such a diet in such an animal.

*Dog B2-86.*—Male; short haired mongrel; cinnamon and black; very old, obese, and phlegmatic, but vigorous and active; weight 32.4 kilos. Apr. 7, 1914. Removal of pancreatic tissue weighing 48.9 gm. Remnant about main duct estimated at 12.4 gm. ( $\frac{1}{2}$ ). The dog was inordinately greedy, but had no glycosuria even from the largest amounts of bread and soup which he ate. Apr. 19. This diet was increased by 250 gm. of glucose, with resultant glycosuria of 1.25 per cent, which steadily diminished to 0.2 per cent on Apr. 23. The glucose was then increased to 500 gm. daily, but nevertheless glycosuria was absent thereafter. It was possible to continue heavy glucose feeding with only a few days of intermission to avoid anorexia, indigestion, or diarrhea. The bread was ordinarily not weighed, but on certain test days the dog eagerly ate as much as 1 kilo of dry bread and 800 gm. of glucose. The average bread ration was nearer 500 gm., but 400 to 600 gm. of weighed glucose were taken with this. No other dog in the series was willing to eat or able to digest such quantities of carbohydrate, and advantage was taken of this peculiarity in order to test whether diabetes might ultimately result from such a load upon the carbohydrate metabolism. Glycosuria was rare and slight. Gain in tolerance was also indicated by tests on May 4 and 13. On each of these dates the dog was fed 400 gm. of bread, 200 gm. of glucose, and 500 cc. of soup, and the urine and plasma sugars were followed as shown in the accompanying table.

### *Dog B2-86.*

Date.	Time.	Plasma sugar. per cent	Urine.		Date. 1914	Plasma sugar. per cent	Urine.	
			Volume. cc.	Sugar. per cent			Volume. cc.	Sugar. per cent
1914								
May 4	Before feeding.	0.089	376	0	May 13	0.099	123	0
	$\frac{1}{2}$ hr. after feeding.	0.196	12	Faint.		0.196	6	0
	1½ hrs. " "	0.286	15	0.52		0.156	5	0
	2½ " " "	0.298	21	0.52		0.192	2 drops	Faint.
	3½ " " "	0.357	14	1.76		0.232	25	0.62
	4½ " " "	0.476	80	3.53				
	6 " " "	0.435	75	3.61		0.285	25	1.22
	8 " " "	0.400	95	3.22		0.270	25	1.13
	9 " " "	0.270	55	2.22		0.286	35	1.00
	11 " " "	0.294	45	1.81		0.250	75	0.55
	13 " " "	0.263	3	0.96				
	14 " " "	0.156	100	0.70		0.176	110	Faint.
	16 " " "	0.099	40	0		0.098	155	0
	24 " " "	0.062	143	0		0.098	320	0

The poor prospect of overcoming the increased tolerance by higher doses of carbohydrate was indicated by a shorter test on July 21.

*Dog B2-86.*

Date.	Time.	Plasma	Urine sugar.	Remarks.
		sugar. per cent	per cent	
1914 July 21	Before feeding.	0.124	0	Fed 400 gm. of bread with 500 gm. of glucose.
	2 hrs. after feeding.	0.147	0	
	7 " " "	0.141	0	

Aug. 7. Removal of a bit of pancreas was attempted. The remnant was excessively congested; stubborn oozing prolonged the operation a little, and the dog died under the operation. The pancreatic congestion was not necessarily associated with the sugar feeding, for it has been found in occasional dogs under other conditions. The easy death from ether or shock may have been due to injury from the glucose, though the animal seemed as well and strong as ever, or merely to senility and obesity.

*Autopsy.*—The pancreas remnant weighed 24.8 gm. and the tissue including the islands was normal in all respects. The hypertrophy of the remnant was thus accompanied by recovery from diabetes to such an extent that no apparent injury resulted from the most excessive gluttony in carbohydrate for 4 months. The intact state of the islands was evidence that more prolonged carbohydrate excess could not have caused a lowering of tolerance, which is due to hydropic degeneration of the islands as formerly described.

*Repeated Operations.*

It is a familiar and readily comprehensible experience that glycosuria may be absent or transitory on the highest possible starch and sugar feeding, and that the diabetes which could not be induced by diet is brought on by the removal of an additional tiny fragment of pancreas. An example was previously given.<sup>4</sup> Others are mentioned in the preceding paper.<sup>5</sup> It is there seen that 0.3 gm. of tissue in the case of Dog C3-45, or 0.22 gm. in the final operation of Dog B2-63, sufficed to make the difference between diabetes and its absence. Certain points will be further developed by giving a summary of individual protocols.

<sup>4</sup> Allen,<sup>1</sup> p. 486, Dog 176.

<sup>5</sup> Allen, F. M., *J. Exp. Med.*, 1920, **xxi**, 375, "Remarks" column of Table IV.

*Dog B2-63.*—Male; mongrel; brown and white, shaggy; age 6 years; slightly thin; weight 24.4 kilos. May 22, 1914. Removal of pancreatic tissue weighing 41.6 gm. Remnant about main duct estimated at 7.4 gm. ( $\frac{1}{2}$ - $\frac{1}{2}$ ). Glycosuria was continuously absent on a diet of beef lung up to June 23. The diet was then changed abruptly to bread and soup. Glycosuria of 0.2 per cent in 1,140 cc. of urine was present on June 24, but ceased immediately. June 29. 100 gm. of glucose were added, with resultant glycosuria of 0.52 per cent in 1,185 cc. of urine; this also ceased immediately. Similar transitory traces of glycosuria followed the increase to 200 gm. of glucose on July 1, to 300 gm. on July 8, and to 400 gm. on July 30. On Aug. 3 (after more than a month of continuous bread and glucose diet) the attempt to produce diabetes by feeding was abandoned, and the dog was used for phlorizin experiments.

Nov. 6. An additional 1.32 gm. of pancreatic tissue was removed, the body weight being then 23.9 kilos. Nov. 23. The urine was still sugar-free on bread and soup diet; 100 gm. of glucose were given by stomach tube before feeding. This procedure caused moderate glycosuria on this day, less on the next day, and none on the following days. Nov. 28. The glucose feeding was stopped. Thereafter on plain bread and soup diet, slight to moderate sugar reactions appeared on Dec. 1, 8, 9, 10, 12, and thenceforth continuously to Jan. 3, 1915. The occurrence of glycosuria at first was governed by the quantities of bread eaten, the dog being hungrier on certain days. This lowering of tolerance on prolonged carbohydrate diet took place notwithstanding a loss of body weight to 22.7 kilos on Dec. 12. Glycosuria ceased with a single fast day on Jan. 3, and thereafter was continuously absent on a diet of beef lung *ad libitum*. Mar. 30. Weight 22 kilos; the only food given was 200 gm. of bread (weighed dry, and moistened with soup before feeding). The result was glycosuria of 0.87 per cent in 860 cc. of urine. Several other tests as mentioned below, to compare the assimilation of different starches, indicated a similar lowering of tolerance. As the dog did not thrive on the monotonous lung diet, the weight gradually fell, reaching its lowest level of 17.9 kilos on June 26. Corresponding to this marked decline in weight was a rise in tolerance, so that 200 gm. of bread no longer caused glycosuria; also on July 6, at a body weight of 18.75 kilos, the sugar in the blood plasma after ingestion of this quantity of bread rose only to 0.143 per cent. The diet was then changed to lung and suet *ad libitum*; the body weight gradually rose to 27 kilos in Aug. and Sept. The dog proved able to take 200 gm. of bread in addition to the usual diet, and on Sept. 20 the diet was changed to bread and soup with suet. Glycosuria remained absent, with the exception of 0.34 per cent on Oct. 7 and 0.32 per cent on Oct. 10. The body weight gradually rose, until by the middle of Nov. the animal was obese at a weight of 30.9 kilos. Nov. 16. Slight glycosuria appeared, and increased on Nov. 17. With 1 day of fasting and 1 day of exercise, glycosuria ceased and remained absent, on a diet of bread and soup, while the body weight fell to 30 kilos.

Nov. 30. Weight 30 kilos; pancreatic tissue weighing 0.36 gm. was removed. During the ensuing week traces of glycosuria occurred on bread and soup diet, though the dog ate poorly. Fasting was then imposed from Dec. 7 to 16.

Dec. 16. Weight 24.25 kilos. The animal was given all the bread and soup he would eat. Heavy glycosuria appeared (hunger glycosuria), diminishing to faint glycosuria on the following day and remaining absent thereafter, except for a trace of 0.14 per cent on Dec. 31. The weight slowly fell to 23 kilos by the middle of Feb.

Beginning on Feb. 14, 1916, at this weight, 200 gm. of glucose were added to the bread and soup diet. On this day there was glycosuria of 0.37 per cent, which ceased immediately. Beginning Feb. 18, 100 gm. of lard were added daily to the bread and glucose diet. The weight rose to 24.7 kilos by Feb. 26, while glycosuria remained absent. On this date the dog was changed from the warm room to an outdoor cage. On the identical diet traces of glycosuria were continuous for a few days, as will be described in a subsequent paper. The weight was meanwhile falling to 23.4 kilos, and on Mar. 4 to 6 an increase to 400 gm. of glucose produced no more than 0.45 per cent glycosuria in 600 cc. of urine.

Mar. 7. Pancreatic tissue weighing 0.22 gm. was removed. Thereafter bread and glucose feeding maintained glycosuria without difficulty. Mar. 27. An additional 0.8 gm. of pancreatic tissue was removed. Apr. 2. Death occurred from peritonitis.

*Autopsy.*—The pancreas remnant weighed 12.3 gm.

Microscopically, the important feature was the existence of vacuolation of island cells throughout the series of pancreatic specimens, very slight and scarce in the operations of Nov. 6, 1914, Nov. 30, 1915, and Mar. 7, 1916, and decidedly more advanced on Mar. 27, 1916, and at autopsy.

The clinical course, especially with consideration of the shorter life of a dog, bears a resemblance to a prolonged mild case of human diabetes. The vicissitudes and fluctuations of tolerance might appear superficially as bizarre and accidental, but comparison with numerous other examples furnishes a rational explanation for each change.

The factors to be observed particularly as influencing the tolerance are the diet, the body weight, and the natural reparative power. In brief, it will be noticed that up to January, 1915, heavy carbohydrate feeding reduced the tolerance, even though the urine was generally sugar-free and even though the body weight fell. This lowering of assimilation was still perceptible on March 30, but with carbohydrate-free diet and further decline of weight, the tolerance in June and July was found higher, and in September the animal was able to live on bread without glycosuria. The body weight was then increased by

the addition of fat to the diet. Evidence of actual reparative power in the pancreas was afforded, for the improved assimilation continued even at higher weights than before. But when in November the animal became obese at a weight above 30 kilos, decided glycosuria appeared. The same combination is evident in the operation of November 30 and the subsequent fast. The loss of 0.36 gm. of pancreatic tissue was more than counterbalanced by the reduction to 24.25 kilos in weight; but the genuineness of the reparative process is proved by the higher tolerance and weight as compared with the preceding March.

The reparative process mentioned may be associated with the marked hypertrophy of the pancreas remnant. The recovery, however, was overbalanced by the subsequent removal of 0.36 and 0.22 gm. of additional pancreatic tissue. The newly formed tissue therefore could not be functionally equivalent to the original tissue.

Even though no obvious clinical injury seemed associated with the carbohydrate excess preceding November 6, 1914, or with the obesity of November, 1915, or with the carbohydrate excess prior to March 7, 1916, the microscopic examination demonstrated injuriously overtaxed island cells at all these times. The downward progress clinically would doubtless have been still more evident if it had not been checked at certain periods as mentioned, and also if the observations had been extended for a still longer time. This downward progress is fully comparable with that of mild human cases treated on the lax plan of merely keeping the urine sugar-free most of the time, and it cannot properly be called spontaneous.

It will bear repeating that experiments of this sort require prolonged careful attention to the diet and all other details. Care must be taken that the animal likes his food and eats it, that he does not suffer from diarrhea or other illness, and that the regularity of his program shall equal that of diabetic patients whom he is supposed to imitate. Even so, only a minority of dogs thrive under the conditions and are adaptable to changes of diet and weight as desired. Therefore they are generally obtainable only by trial and choice among a considerable series of dogs. The best ones, which prove capable of such a delicately balanced tolerance and such close reproduction of clinical conditions, represent much labor and are useful for many experiments.

*Dog C3-27.*—Female; mongrel; yellow; age 4 years; good condition; weight 16.25 kilos. July 8, 1915. Removal of pancreatic tissue weighing 34 gm. Remnant about main duct estimated at 4.4 gm. ( $\frac{1}{4}$ ). The subsequent condition was mild diabetes. Glycosuria was repeatedly produced by feeding 50 or 100 gm. of bread, or on certain occasions by large diets of beef lung. Also at the time of these tests the weight was down to approximately 15 kilos. Beginning Oct 1, the diet was 500 gm. of beef lung and suet *ad libitum*. Dec. 1 to 5. The addition of 100 gm. of bread caused daily glycosuria not above 0.42 per cent in 450 cc. of urine, the weight then being 19.8 kilos. On omitting the bread the glycosuria ceased. Beginning Feb. 14, 1916, the addition of 100 gm. of bread produced no glycosuria. Feb. 23. The diet was changed to bread and soup. Feb. 25. 100 gm. of glucose were added to this diet, causing glycosuria of 0.63 per cent in 940 cc. of urine for 1 day. Glycosuria then ceased and could not be restored, though the glucose dosage was 200 gm. on Feb. 27, 300 gm. daily from Feb. 28 to Mar. 4, and 400 gm. daily to Mar. 9, when the attempt had to be abandoned because of distaste for the bread and glucose mixture. The dog meanwhile had become obese, reaching the maximum weight of 21 kilos.

Mar. 9. An additional 0.9 gm. of pancreatic tissue was removed. Bread diet caused no glycosuria on the following days. Beginning Mar. 13, 200 gm. of glucose were added daily, with negative urine till Mar. 16, when heavy glycosuria suddenly appeared. This continued after glucose was discontinued on Mar. 21. The subsequent course was downward progress on mixed diet to death in coma on Apr. 22.

*Autopsy.*—The pancreas remnant consisted of normal appearing tissue weighing 7.8 gm. Islands of Langerhans were very large and abundant but maximally vacuolated.

In the tissue removed at operation on Mar. 9, the islands were free from any visible abnormality.

Genuine recuperation of function in the pancreas remnant was demonstrated by the double evidence of markedly increased carbohydrate tolerance at a higher body weight, up to March 9. In view of the facts that the dog's digestion could not bear further prolongation of the high glucose feeding, that the islands at that time showed no sign of vacuolation, and that the tendency to recovery was so manifest, it is improbable that diabetes could have been brought on by any kind or duration of feeding.

This recovery of function corresponded to a marked hypertrophy of the pancreas remnant, which doubled in size. The great abundance of islands found in numerous slides indicated that they underwent hyperplasia which fully paralleled that of the acini. Nevertheless, the functional recovery was completely negatived by the removal of

only 0.9 gm. of additional tissue. The new formed tissue was therefore not equal to the original tissue in functional capacity.

The reason for the recovery of assimilative power, to such an extent that a second operation was required, lay in the long interval of sugar freedom granted to the animal after the first operation. If the pancreas remnant is of the large size which is most desirable, a dog should be subjected to occasional periods of glycosuria to prevent the tolerance from rising too high. Only after the diabetic condition has been thus maintained for months or years can the animal be trusted to remain permanently diabetic without this precaution. The existence of a considerable recuperative power at the outset, and the feebleness or absence of this power in the later stages, correspond closely to the well known facts in human diabetes.

By comparison with numerous other dogs, it may safely be assumed that carbohydrate overfeeding after the first operation would have sent this animal rapidly into severe diabetes. A carbohydrate-free diet, with limited protein so as to keep the urine sugar-free, permitted recovery of tolerance, even though the fat ration was so high as to produce obesity. Provided that a recuperative power exists in the pancreas, such a diet evidently affords opportunity for recovery and is far less harmful than an excessive carbohydrate diet. This fact was abundantly demonstrated by the benefits of the classical treatment in human cases, and is confirmed by similar observations in dogs.

*Dog C3-45.*—Female; mongrel; yellow; age 4 years; moderately well nourished; weight 10.8 kilos. Nov. 19, 1915. Removal of pancreatic tissue weighing 23.8 gm. Remnant about main duct estimated at 3.7 gm. ( $\frac{1}{2}$ - $\frac{1}{4}$ ). Glycosuria remained absent with fasting up to Nov. 23. Feeding of bread and soup then brought on heavy glycosuria. Nov. 27. The diet was changed to beef lung; glycosuria progressively diminished, and was absent on Nov. 30. Dec. 1. Heavy sugar (4.45 per cent in 380 cc. of urine) returned on bread diet, then stopped after 2 days of lung diet. Dec. 6. The dog was loaned to another laboratory, where she remained for a time on diets which caused little or no glycosuria. Mar. 16, 1916. The dog was sent back with the report that she was not diabetic. On this date the feeding of bread and soup with 300 gm. of glucose caused excretion of 1.6 per cent sugar in 528 cc. of urine; but the appetite and digestion of the dog could not endure continuance of this sugar dosage, and glycosuria therefore remained absent till Mar. 27. The body weight during this time was 11.3 to 11.6 kilos.

Mar. 27. Pancreatic tissue weighing 0.3 gm. was removed. Glycosuria remained absent with fasting for 2 days. Mar. 29. Bread and soup diet caused faint glycosuria, which became heavy (4.4 per cent in 2,000 cc. of urine) on the addition of 50 gm. of glucose. After Apr. 2, it continued heavy on plain bread and soup diet, and the dog was kept in a condition bordering on glycosuria thereafter. Glycosuria was absent on restricted diet, but always returned promptly on feeding bread or too much meat, and there was no further tendency to any marked recovery of tolerance. The animal was sent to another laboratory on July 10.

The noteworthy feature is that the considerable recovery of tolerance in this case was overbalanced by the removal of 0.3 gm. of additional tissue, and that this tiny bit of pancreas made the difference between diabetes and its absence.

*Dog C3-86.*—Female; mongrel; brown; age 3 years; good condition; weight 15 kilos. Apr. 28, 1916. Removal of pancreatic tissue weighing 33 gm. Remnant about main duct estimated at 2.9 gm. ( $\frac{1}{3}$ — $\frac{1}{3}$ ). Appetite was poor and weight was lost rather rapidly after operation. Glycosuria was absent on bread and soup eaten in small quantities, but present on the addition of 50 gm. of glucose. Sugar freedom on bread diet continued till May 22. Thereafter, 100 gm. of glucose were given by stomach tube before feeding each day. There was glycosuria of 1.8 per cent in 670 cc. of urine on May 23, 0.42 per cent in 312 cc. of urine on May 24, and none thereafter. Failing appetite compelled the stopping of glucose on June 11. Perhaps because of larger eating of bread and soup, glycosuria reappeared on June 15, the body weight being 13.5 kilos.

On that date 0.2 gm. of pancreatic tissue was removed, and at operation it was estimated that no hypertrophy of the remnant had occurred. The specimen was normal microscopically except for vacuolation in a very few cells of the abundant islands. Subsequently the dog proved vigorous up to death on Dec. 12, 1917. The pancreas remnant was soft and appeared normal, and its low weight of 1.6 gm. was perhaps partly due to the fact that emaciation had reduced the body weight to 6.4 kilos.

The apparently high tolerance in this dog was due to poor eating and loss of weight. For the best diabetic experiments it is essential that the dogs should have good appetite and health in other respects.

*Dog C3-98.*—Male; mongrel; brindle; age 2 years; moderately well nourished; weight 13.4 kilos. June 8, 1916. Removal of pancreatic tissue weighing 25.8 gm. Remnant about main duct estimated at 2.1 gm. ( $\frac{1}{3}$ ). Diabetes was controlled by diet, but its existence was demonstrated by the fact that during 2 months glycosuria could be produced at any time by feeding 100 gm. of bread.

The tolerance was spared by diet until Nov., when the body weight ranged slightly above and below 12 kilos. Bread and soup diet then failed to bring on glycosuria, and during the month Nov. 21-Dec. 21 the daily addition of 200 or 300 gm. of glucose also failed.

Dec. 21. Additional pancreatic tissue weighing 0.85 gm. was removed, the body weight being 11.25 kilos. The dog was unwell thereafter, with diarrhea and poor appetite, so that by Jan. 5, 1917, the body weight had fallen to 9.5 kilos. Glucose feeding as high as 300 gm. daily again failed to produce more than transitory glycosuria, though the dog began to regain weight. Feb. 7. Weight 11.4 kilos; the plasma sugar at 11 a.m. was 0.112 per cent. The usual bread and soup mixture with 300 gm. of glucose was then fed; glycosuria was absent, and at 4 p.m. the plasma sugar was 0.111 per cent.

Feb. 8. 0.5 gm. of additional pancreatic tissue was removed. Diabetes existed thereafter, so that glycosuria could be produced by plain bread and soup diet. June 4. Died; the dog was emaciated down to 8.6 kilos, and the pancreas remnant weighed 2.95 gm.

In the tissue removed Dec. 21, vacuolation of island cells was rare if present, and so slight as to be doubtful. Also on Feb. 8, no positive changes in the islands were demonstrated. The emaciation preceding death was due to other causes than diabetes, and there was no vacuolation of islands.

The long interval of freedom from glycosuria following the first operation permitted actual recovery from the diabetes. This was associated with hypertrophy of the pancreas remnant, and the new formed tissue seemed to be functionally equivalent to the old, for further resection was necessary practically to the same extent as in an original operation, even for an animal at the lower body weight. As usual, carbohydrate overfeeding did not cause degenerative changes in the islands of the non-diabetic animal, and the potential diabetes subsequently in absence of active symptoms was also attended with no island changes.

#### *Glucose Intoxication.*

Glucose overfeeding ordinarily causes no harm in dogs beyond diarrhea and loss of weight. In exceptional instances the attempt to produce diabetes has ended in death apparently from poisoning by the prolonged excess of sugar. Gastrointestinal disturbances have appeared sometimes as a prominent cause, and sometimes have been absent or trivial. Though these cases in such a resistant species as dogs are not positive, the genuineness of glucose intoxication is confirmed by fatalities in feebler animals, such as rabbits and monkeys, and in dogs weakened by a preceding Bernard puncture of the medulla.

*Dog B2-48.*—Female; mongrel; yellow and white; age 4 years; good condition; weight 14.75 kilos. Mar. 19, 1914. Removal of pancreatic tissue weighing 22.5 gm. Remnant about main duct estimated at 4.7 gm. ( $\frac{1}{2}$ ). Glycosuria being absent on bread and soup diet, on Mar. 30, 200 gm. of glucose were added; the amount was increased on Apr. 1 to 300 gm. As the dog did not like this mixture, on Apr. 9 a mixture of chopped meat, bone-meal, and glucose was substituted, the latter in dosage of 200 or oftener 300 gm. daily. The dog held weight, without glycosuria, and seemed to be thriving until she was unexpectedly found dead on May 9.

*Autopsy.*—No cause of death was found and no organ changes beyond intense congestion of the liver. Though the animal had been dead several hours, 100 gm. of liver were taken in boiling potassium hydroxide, but the concentrated extract was negative for glycogen. Microscopically, the pancreas remnant (weight 6.8 gm.) and its islands were normal; the liver was strictly normal, except for intense congestion, and was not fatty; the other organs were negative. Death was due to some unknown intoxication.

*Dog B2-69.*—Male; bulldog; mongrel; white with black head; age 4 years; good condition; weight 19.9 kilos. May 29, 1914. Removal of pancreatic tissue weighing 36.3 gm. Remnant about main duct estimated at 9.8 gm. ( $\frac{1}{2}-\frac{1}{4}$ ). The dog thrived on bread and soup diet. July 8, 100 gm. of glucose were added, increased on July 15 to 200 gm., on July 20 to 300 gm., and on July 30 to 400 gm., the mixture being improved by the addition of a little chopped meat daily. The weight gradually diminished, but the dog remained lively. Oct. 20. Weight 14 kilos; there was weakness out of all proportion to the emaciation. The animal, though retaining appetite and spirits, could not stand. Glucose was discontinued and meat fed. Oct. 22. The dog was still unable to stand, though wagging his tail and making efforts to rise. On this day was noticed bloody diarrhea, which previously had either been absent or too slight to attract attention. Oct. 23. The animal was found dead.

*Autopsy.*—The pancreas remnant appeared normal and weighed 16.1 gm. The stomach and intestines appeared normal throughout except for a portion 3 feet in length beginning 6 feet from the pylorus, where the mucous membrane was deeply injected and ecchymotic. The liver and other viscera appeared normal without congestion. There was no noticeable excess or diminution of fluid in the brain or cord. Microscopically the liver, adrenals, and pancreas were found normal. As in Dog B2-48, no fat vacuoles were visible in routine stains of the liver.

Dog B2-49, subjected to similar prolonged glucose feeding, died of gas bacillus infection, which is rare in dogs. Experiments with infection of diabetic dogs with this organism will be published later, but were on the whole negative.

*Comparisons of Carbohydrates.*

*Comparison of Starch and Glucose.*—Numerous observations have made it evident that glucose brings on glycosuria and diabetes more actively than starch. The difference is not merely one of quantity, for the addition of 50 or 100 gm. of glucose is sometimes effective when no amount of bread feeding avails for glycosuria. The reason for the readier glycosuria from glucose lies evidently in its quicker absorption, and if distributed in sufficiently small doses throughout the day it would presumably be assimilated as well as starch, as Klemperer showed in human patients. The most important point here is that the sudden glucose flood with its attendant glycosuria is more injurious to the pancreatic function than the more gradual and prolonged labor imposed by starch. It may thus be inferred that sugar is a more dangerous food for human beings with any predisposition to diabetes than is starch.

The question may be raised whether the difference is not merely one of time, and whether starch will not bring on diabetes more slowly than glucose but just as surely. As the difference between the two is not very great, experiments need to be performed with full precautions that the body weight does not vary, that the preliminary tests of the glucose tolerance do not lower the tolerance for starch, and under other conditions of exactness. Examples have already been given in which bread feeding was tolerated for several days before onset of glycosuria. The feature which renders longer tests peculiarly difficult is the recuperative power of the pancreas, which through regeneration reduces the diabetic tendency sometimes to the vanishing point, as described in preceding protocols. This experimental difficulty strengthens the view of the greater danger of sugar for predisposed human individuals, because such power of regeneration as their pancreas may possess will evidently have a better opportunity on starch diet. The difficulty may by extreme care be overcome in very prolonged experiments, in which the animals are kept potentially diabetic for so long a time that the regenerative tendency largely disappears. These conditions were fairly well fulfilled in Dog B2-02, described above. This animal could have been made actively diabetic at any time by glucose except for her repugnance to it. The

feature of incompleteness in the experiment is that death occurred from rabies before diabetes actually ensued on starch diet, but the hydropic degeneration in the islands of Langerhans demonstrated sufficiently that loss of tolerance was in progress and diabetes inevitable.

The record of Dog B2-01 from Sept. to Dec., 1916, to be published later, also shows a downward progress on bread diet, though the diet at first was apparently assimilated even with the addition of 300 gm. of glucose. The same result is shown in the experiments with Dogs D4-52 and D4-69 in Paper 3 of this series.<sup>6</sup> In the former animal between Aug. 8 and 21, 1917, it was impossible to maintain glycosuria on bread diet with 200 gm. of glucose, and the sugar feeding had to be stopped because of the distaste acquired by the dog for it. The tests of blood and urine on Aug. 8 and 15 also indicated a rise of tolerance during this period. But with continuance of plain bread feeding, glycosuria appeared on Oct. 10, and in a test with the addition of 200 gm. of glucose to the usual bread mixture the excretion was as high as 7 per cent. Likewise Dog D4-69 displayed an apparently increasing assimilation, so that the animal finally tired of glucose and the attempt to maintain glycosuria apparently failed. But with continuance of bread feeding, glycosuria began on Oct. 6, and, just as with Dogs B2-01 and D4-52, the tolerance proved to be permanently lowered thereafter.

When the regenerative power of the pancreas is not so great as to result in a practical cure of the diabetes, the difference between starch and glucose seems to be essentially one of time and degree. Glucose through its quicker absorption floods the body more suddenly and violently, but starch works the same damage more slowly but just as surely. This was shown in several animals through the onset of diabetes, and in one by examination of the islands of Langerhans. By inference, if human diabetes is so mild that glycosuria ceases on withdrawal of sugar without limitation of other carbohydrate, it is not correct to treat the case in this manner or to leave either starch or total calories unrestricted.

When the regenerative power of the pancreas is sufficient, there is a real difference due to the time element mentioned, and a number of examples have already been given of more or less complete recovery from diabetes on starch diet by dogs which could readily have been made diabetic by forcing of glucose shortly after operation. Some-

<sup>6</sup> Allen, F. M., *J. Exp. Med.*, 1920, **xxx** (in press).

thing similar may conceivably occur in some human patients. In properly chosen animals without too strong a tendency either to diabetes or to recovery, it is possible to demonstrate a recuperative attempt by functional tests showing an apparent increase of assimilation, but the continued overtaxing of the assimilation causes its breakdown, and recovery to the former extent is never again possible. A similar gain of tolerance is well known in human patients under dietary restriction in the earlier stages of diabetes, and the warning against abusing this recuperative tendency is similar, especially as actual recovery on the part of human patients has seldom been witnessed.

Three of the four dogs here mentioned were kept sufficiently long to demonstrate that the lowering of tolerance referred to was actually the result of the carbohydrate excess and not of any inherent tendency in the dogs. The onset of diabetes was evidently checked by change to protein-fat diets, and especially in Dog B2-01 the tolerance could be manipulated up and down by changing the body weight. It is therefore confirmed that carbohydrate injures the assimilation more rapidly and powerfully than any other food. The later observations show also the harmfulness of luxus diets, but these diets are most dangerous when they include carbohydrate.

#### *Comparisons of Starches.*

Experiments were begun to test the glycosuric effect of different starchy foods, in relation to the hypotheses of certain writers concerning carbohydrate "cures." Dogs with potential diabetes of long standing and with fairly stationary assimilative power were given quantities of carbohydrate corresponding to the limits of their known tolerance. Stale bread was used, with a carbohydrate content roughly similar to that of the cereals. The bread was moistened with water after weighing. The cereals were weighed raw, and then boiled. No other food was given on the test days, and a fixed diet of 1 kilo of beef lung was followed on all other days. There was never glycosuria except on test days.

On test days approximately 50 gm. of starch were administered to Dog B2-43 in the following forms:

*Dog B2-43.*

Date.	Food.	Glycosuria.
1914		gm.
Sept. 10	Oatmeal.	0
" 15	Rice.	5.5
Oct. 2	Potato.	0
" 10	Bread.	6.4
" 19	Pearled barley.	2.3

The program was continued in the same animal by varying the quantities of the different foods, with a view to testing the tolerance for each.

Date.	Diet.	Glycosuria
1915		gm.
Mar. 26	75 gm. oatmeal.	0
" 30	100 " "	0
Apr. 1	200 " "	0.7
" 16	50 " bread, 1 kilo lung.	Faint.
" 19	50 " " 1 " "	0
" 20	100 " " 1 " "	Slight.
" 24	100 " "	3.0
" 27	100 " oatmeal.	0
May 3	100 " "	0
" 5	150 " "	0
" 10	200 " "	Faint.
" 17	200 " " 1 kilo lung.	0
" 25	200 " rice.	8.4
June 1	100 " "	6.4
" 8	50 " "	Faint.
" 15	200 " oatmeal.	3.4
" 26	200 " rice.	1.6

*Dog B2-63.*

Date.	Diet.	Glycosuria.
1915		gm.
Apr. 16	50 gm. bread.	0
" 20-23	100 " " 1 kilo lung daily.	Traces.
" 24	150 " " 1 " "	1.6
" 27	150 " oatmeal.	0
May 3	150 " "	2.6
" 10	200 " "	2.3
" 17	200 " " 1 kilo lung.	1.1
" 25	200 " rice.	5.6
June 1	100 " "	11.7
" 8	50 " "	Trace.
" 15	200 " oatmeal.	"
" 25	200 " rice.	0
July 3	200 " bread.	0
" 6	200 " "	0

*Dog B2-71.*

Date.	Diet.	Glycosuria. gm.
1915		
Mar. 29	100 gm. bread.	0
" 30	200 " "	11.4
" 31	Fast day.	0
Apr. 1	300 gm. bread.	6.8
" 16	50 " " 1 kilo lung.	0
" 20	100 " " 1 " "	0
" 24	100 " "	1.4
" 27	100 " oatmeal.	0
May 3	100 " "	Trace.
" 5	150 " "	0
" 10	200 " "	8.7
" 17	200 " " 1 kilo lung.	9.1
" 25	200 " rice.	19.3
June 1	100 " "	18.7
" 8	50 " "	0
" 15	200 " oatmeal.	0
" 26	200 " rice.	2.2

*Dog B2-76.*

On test days approximately 50 gm. of starch were administered as follows:

Date.	Food.	Glycosuria. gm.
1914		
Sept. 10	Oatmeal.	Trace.
" 15	Rice.	3.1
Oct. 2	Potato.	1.1
" 10	Bread.	2.8
" 19	Pearled barley.	1.2

*Dog B2-81.*

Date	Diet.	Glycosuria. gm.
1915		
Mar. 31	200 gm. bread.	0
Apr. 1	300 " "	0.73
" 16	50 " "	0
" 19	50 " " 1 kilo lung.	0
" 20	100 " " 1 " "	2.8
" 24	100 " "	0.16
" 27	100 " oatmeal.	0
May 3	100 " "	0
" 5	100 " "	0
" 10	200 " "	0
" 17	200 " " 1 kilo lung.	3.8
" 25	200 " rice.	5.3
June 1	100 " "	7.4
" 8	50 " "	0
" 15	200 " oatmeal.	Trace.

The claims for the superior assimilability of certain starchily foods, particularly oatmeal, have been previously reviewed.<sup>7</sup> As the claims included some observations with totally depancreatized dogs, the above tests were begun upon partially depancreatized animals, which more closely resemble human diabetics in digestive power and other respects. They were intended as orientation experiments, to decide whether an investigation with combined analyses of the food, feces, and blood sugar was worth while. The following deductions were made.

Precautions were used against fluctuations of assimilation due to changes of body weight, indigestion, diet in the intervals between test days, and other known factors. Dog B2-63 obviously gained tolerance toward the close of the experimental period; otherwise there was success in the attempt to choose animals with fairly stationary tolerance. In Dog B2-71, a single fast day on March 31 evidently increased the bread tolerance considerably, as shown by comparison of March 30 and April 1. With all care against disturbing influences, the tolerance of the animals shows occasional fluctuations from unknown causes, which would seriously hamper accurate experiments upon such test objects. The milder cases of human diabetes, which have been used for comparisons of carbohydrate tolerance, are subject to fully as great fluctuations of tolerance as these animals, and the exactness of tests upon them is open to similar question.

Owing to such accidental fluctuations, the assimilation of oatmeal might rarely seem inferior to that of rice, as in Dog B2-43 on June 15 and 26. But the number of observations was great enough to establish the rule that glycosuria from oatmeal was in general much less than that from any other carbohydrate. Generally the dogs ate the oatmeal less willingly than other foods, notably bread and rice, which caused greater glycosuria. Even when it was eaten promptly, and in the absence of diarrhea, there is the usual question whether the absorption is actually equal, and the recognized impossibility of de-

<sup>7</sup> Allen,<sup>1</sup> Chapter IX. Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, Chapter I.

termining the rate of absorption or the extent of fermentation by fecal analyses or any other means. The above findings in dogs are similar to the clinical observations on which the oatmeal treatment was based, but the calorimetric studies of Du Bois<sup>8</sup> confirmed the more modern view that the assimilation of oatmeal is not actually superior to that of other starchy foods. When this more accurate method became available, the animal experiments were dropped.

Carbohydrate fed at the same time with protein in the form of beef lung sometimes caused slightly greater glycosuria than the carbohydrate alone. For example, Dog B2-71 showed slight differences of this character with oatmeal on May 10 and 17, and Dog B2-81 with bread on April 20 and 24, and with oatmeal on May 17 and June 15. But in equally numerous instances it is seen that the glycosuria was greater from carbohydrate alone (Dog B2-43, April 20 and 24, May 10 and 17; Dog B2-63, May 10 and 17; Dog B2-71, April 20 and 24), as if the mixture with protein had slowed the absorption of the carbohydrate. The observations are entirely contrary to the claim that the assimilability of oatmeal is spoiled by simultaneous ingestion of meat.

#### CONCLUSIONS.

1. The injurious effects of excessive carbohydrate diet are demonstrable in partially depancreatized dogs, in the same manner as in human patients. With severe diabetes there is rapid progress of emaciation and weakness and early death.

2. With milder diabetes, there is frequently a transitional state following operation, when the fate depends on the diet. If the tolerance is spared for a time, recovery sometimes occurs to such extent that diabetes cannot be produced by any kind or quantity of feeding, but only by removal of a small additional fragment of pancreatic tissue. The proper degree of carbohydrate overfeeding is important in this early period for producing the most useful type of diabetic animals; namely, those having good digestion and general health combined with a permanent lowering of assimilative power, like the condition of human patients.

<sup>8</sup> Allen, F. M., and Du Bois, E. F., *Arch. Int. Med.*, 1916, xvii, 1010.

3. In the early stage, glucose is more powerful than starch in producing diabetes, and animals which are progressing toward complete recovery on starch diet can be sent into hopeless diabetes by admixture of glucose. The difference seems to be merely of the rate of absorption, and indicates that a rapid flood of carbohydrate is more injurious to the pancreatic function than a slow absorption. Whenever permanent diabetes is present, so that complete recovery is impossible, starch brings on glycosuria more slowly than sugar, but just as surely. The difference in time in different cases amounts to days, weeks, or months. The clinical lesson from such experiments is that even if a patient becomes free from glycosuria on withdrawal of sugar only, nevertheless other foods should also be limited.

4. No significant differences were observed between the assimilation of different starches, or any extreme lowering of the carbohydrate tolerance by proteins, such as alleged by certain writers in connection with the "oatmeal cure."

5. Repair of traumatic inflammation and hypertrophy of the pancreas remnant have been mentioned incidentally as the basis of the early tendency to recovery, and also hydropic degeneration of Langherans islands as an accompaniment of the lowering of tolerance by excessive diet. These are believed to have their parallels in human cases, and are to be described more fully hereafter.

## EXPERIMENTAL STUDIES ON DIABETES.

### SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG. 3. EFFECTS OF PROTEIN DIETS.

BY FREDERICK M. ALLEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 8, 1919.)

#### *Comparison of Carbohydrate and Protein.*

In mildly diabetic dogs the starch of 50 to 100 gm. of bread or cereal is more active in producing glycosuria than the larger quantity of potential carbohydrate represented in a kilo of beef lung.<sup>1</sup> Undoubtedly the starch would be much better assimilated if distributed in small doses over a longer time, but the supposition that the difference is only one of rapidity of absorption and metabolism is opposed by the following facts: (1) The glycosuria from starch in these animals generally lasts over 6 or even 12 hours, while phlorizin and respiration experiments prove that protein requires no longer time for absorption and deamination. (2) The effect of carbohydrate is often cumulative. For example, Dog B2-89 was free from glycosuria on 1 kilo of beef lung daily. On June 25, 50 gm. of bread were substituted for 250 gm. of the lung. The urine remained negative till a glycosuria of 0.75 per cent appeared on June 30 and 0.71 per cent on July 1. Then on return to the diet of 1 kilo of lung, the urine remained negative up to the following test in August. On August 5, 25 gm. of bread were substituted for 150 gm. of lung. A trace of glycosuria appeared on August 10, and 0.18 per cent on August 14. On August 15, nothing was fed but 50 gm. of bread, and an excretion of 1.4 per cent sugar in 140 cc. of urine resulted. On resumption of the diet of 1 kilo of lung glycosuria remained absent up to the time of another experi-

<sup>1</sup> Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 397-399.

ment on September 17. (3) Blood sugar analyses to be reported later show that protein causes little hyperglycemia in mild diabetes, but in severe diabetes it produces a blood sugar curve resembling that of carbohydrate in milder cases, not only in height but also in rapidity of rise, thus confirming the statement (1) above concerning time relations.<sup>2</sup> Also the maximal dextrose-nitrogen ratios in the severest diabetes are a familiar proof that the carbohydrate of protein is excreted quantitatively and is as incapable of assimilation as pre-formed carbohydrate.

These facts conform to clinical observations and justify the earlier treatment of diabetes, from the time of Rollo onward, inasmuch as the restriction of preformed carbohydrate is after all the foundation stone of dietotherapy, and protein restriction comes secondary in both time and importance.

This simple point also has relation to the question of whether diabetes is a deficiency of utilization of glucose primarily and specifically or of other foods as well. The progressive impairment of protein metabolism, from the stage where protein is apparently assimilated almost perfectly to the stage where it causes hyperglycemia and glycosuria almost identical with those from preformed carbohydrate, has an important bearing upon this question but does not in itself furnish a decisive answer.

#### *Comparisons of Proteins.*

*Dog B2-29.*—This dog, having severe diabetes and a very low tolerance, with a remnant of approximately  $\frac{1}{2}$  of the pancreas, was used for feeding tests with approximately 10 gm. of protein in different forms (Table I).

Tests with different forms of protein in other animals gave similar results. Also Dog B2-25, possessing  $\frac{1}{2}-\frac{1}{3}$  of the pancreas, was used for a more thorough test of pancreas feeding. The body weight was 11.3 kilos on July 4, 1914; 500 gm. of fresh raw beef pancreas were fed daily until July 18, then 600 gm. daily till July 28, then 750 gm. of pancreas till Aug. 2, when the body weight was 14.4 kilos. Slight glycosuria was present from July 30 to Aug. 2. Fasting from Aug. 2 to 10 reduced the body weight to 12.5 kilos. A diet of 500 gm. of beef lung was then given daily to Aug. 18, 600 gm. daily to Aug. 27, 750 gm. daily to Sept. 28,

---

<sup>2</sup> Cf. one example already reported, Allen F. M., *Am. J. Med. Sc.*, 1917, cliii, 362, Chart 8, Dog 386.

800 gm. daily to Oct. 10, 1,000 gm. daily to Oct. 17, 1,200 gm. daily to Oct. 21, and 1,400 gm. daily to Nov. 28. Up to Oct. 29, the lung was boiled after weighing; after that date it was fed raw. Glycosuria was absent till Nov. 25, when 4.1 gm. of sugar were excreted, increasing to 8.2 gm. on Nov. 27. The body weight at this time was 17 kilos. The reason for the higher assimilation of beef lung at a higher body weight was that the dog was gaining tolerance with time. Evidently there is nothing in raw pancreas which alters the natural customary progress of this improvement.

TABLE I.

*Dog B2-29.*

Date.	Diet.	Glycosuria. gm.
1913		
Dec. 29	50 gm. of raw beef.	0
" 31	30 " " soy beans (boiled).	0 2
1914		
Jan. 1	70 " " egg (approximately 2 small eggs), raw.	Trace.
" 2	100 " " lard (no protein)	0
" 3	50 " " raw pancreas.	0 16

Several experiments for comparison of raw and cooked meat (ordinary beef for boiling or beef lung) are typified by the following example.

*Dog D4-74.*—Female; mongrel; age 4 years; moderately well nourished. Jan. 17, 1917. Partial pancreatectomy leaving remnant estimated at  $\frac{1}{4}$ . Weight throughout feeding experiments 13.2 to 13.5 kilos. The quantities of beef lung mentioned (Table II) were either boiled after weighing or fed raw.

The literature to which these experiments are related has been reviewed previously.<sup>3</sup> It was confirmed in numerous observations<sup>4</sup> that after a period of carbohydrate-free diet or especially of fasting the feeding of carbohydrate causes glycosuria, which ceases on continuance of the same carbohydrate diet. Apparently some state of unpreparedness of the body for the sudden flood of carbohydrate is

<sup>3</sup> Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 442, 531, 813-815.

<sup>4</sup> Cf. Dog B2-63, Mar. 30 and Dec. 16, 1915 (Allen,<sup>1</sup> p. 387); also Paper 4 of this series (*J. Exp. Med.*, 1920, xxxi, 576).

TABLE II.  
Dog D4-74.

Date.	Diet.	Glycosuria.
1917		gm.
Apr. 16	400 gm. of cooked lung; 100 gm. of suet.	0
" 17	500 " " " " 100 " " "	0
" 18	600 " " " " 100 " " "	0
" 19	600 " " " "	0
" 20	600 " " raw "	0
" 21	700 " " " "	0
" 22	700 " " cooked "	0
" 23	800 " " " "	0
" 24	800 " " " "	0.8
" 25	800 " " raw "	Faint.
" 26	800 " " cooked "	"
" 27	800 " " raw "	0
" 28	900 " " cooked "	Very faint.
" 29	900 " " raw "	0.25
" 30	900 " " cooked "	Faint.
May 1	1,000 " raw "	Slight.
" 2	1,000 " cooked "	Faint.
" 3	1,000 " " " "	Very faint.
" 4	1,000 " " " " 25 gm. of bread.	10.9
" 5	1,000 " " raw " " 25 " " "	6.7
" 6	1,000 " " " "	5.1
" 7	1,000 " " " "	Slight.
" 8	1,000 " " cooked "	0.5
" 9	1,000 " " raw "	2.2
" 10	1,000 " " cooked "	Faint.
" 11	1,000 " " raw "	1.9
" 12	1,000 " " cooked "	Faint.
" 13	1,000 " " raw "	"
" 14	1,000 " " " "	0
" 15	1,000 " " cooked " " 25 gm. of bread.	Faint.
" 16	1,000 " " raw " " 25 " " "	3.7
" 17	1,000 " " " "	Faint.
" 18	1,000 " " cooked " " 25 gm. of bread.	3.6
" 19	1,000 " " raw " " 25 " " "	6.3
" 20	1,000 " " cooked " " 25 " " "	0
" 21	1,000 " " raw " " 25 " " "	5.7
" 22	1,000 " " cooked " " 25 " " "	8.2
" 23	1,000 " " raw " " 25 " " "	2.9
" 24	1,000 " " cooked " " 25 " " "	3.4
" 25	1,000 " " raw " " 25 " " "	4.1
" 26	1,000 " " cooked " " 25 " " "	0
" 27	1,000 " " raw " " 25 " " "	2.7
" 28	1,000 " " cooked " " 25 " " "	2.2
" 29	1,000 " " raw " " 25 " " "	1.3
" 30	1,000 " " cooked " " 25 " " "	Very faint.
" 31	1,000 " " raw " " 25 " " "	Faint.

here represented, just as later authors<sup>5</sup> have proved that a dose of sugar somehow prepares the normal organism so that a second dose is more perfectly assimilated.

Parenteral injection of pancreas extract may lower the sugar in blood or urine, like various other causes of intoxication or prostration, but no therapeutic benefit has ever been found from such treatment.<sup>6</sup> The feeding of pancreas has never benefited diabetes, either in human patients<sup>7</sup> or in dogs. Dogs are of value for these tests because they can eat much more in proportion to the body weight than human patients, but pancreas in either large or small quantity is found to produce glycosuria as readily as any other form of protein. Sandmeyer's observation that, when pancreatic juice is lacking, the improvement of digestion resulting from pancreas feeding may markedly increase glycosuria has been confirmed by Homans,<sup>8</sup> and serves further to discredit the therapeutic usefulness of pancreas or pancreatic preparations given by mouth. Reach's claim that any raw meat may have the same glycosuric influence as pancreas, through some "toxic" action, appears confusing. The necessity of close personal supervision of feeding experiments by the investigator may again be mentioned. Some dogs have a strong repugnance for raw meat; others are ravenous for raw meat and will scarcely touch cooked meat. Fickleness of appetite and digestion is especially to be watched for in the Sandmeyer type of diabetes. The most plausible assumption to explain Reach's results is that his dogs either refused or vomited the cooked meat. The above experiments prove that there is no appreciable difference between cooked and raw meat in regard to either the glycosuria resulting directly from them or their influence on the assimilation of carbohydrate.

<sup>5</sup> Hamman, L., and Hirschman, I. I., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 306.

<sup>6</sup> Allen,<sup>9</sup> pp. 813-819, 855-857. Kleiner, I. S., and Meltzer, S. J., *Proc. Nat. Acad. Sc.*, 1915, i, 338.

<sup>7</sup> Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, Chapter IV.

<sup>8</sup> Homans, J., *J. Med. Research*, 1915, xxxiii, 1.

Several experiments failed to show any sign of the wide glycosuric differences between proteins alleged by some authors in connection with the oatmeal "cure." There might be some possible interest in exact determinations of blood and urine sugar following the feeding of proteins differing in their content of sugar-forming and non-sugar-forming amino-acids, but the difficulties and uncertainties mentioned with regard to carbohydrate tests are still greater here. The above orientation experiments sufficed to exclude any differences of therapeutic importance.

### *Results of Immediate Protein Excess.*

*Dog B2-56.*—Female; mongrel; white with brown patch over left eye; age 5 years; good condition; weight 16.5 kilos. Apr. 29, 1914. Removal of pancreatic tissue weighing 24.1 gm. Remnant about main duct estimated at 4.2 gm. (†). There was glycosuria for 3 days without feeding, probably because dissection about the pancreas remnant was followed by inflammation in it. Subsequently there was heavy continuous glycosuria on meat diet. May 14. Two tiny bits of tissue, weighing together only a small fraction of a gram, were removed from the pancreas remnant for examination. The remnant at this time did not appear on gross inspection as inflamed. Fasting was then imposed, May 14 to 23, but glycosuria was not reduced below 1.3 per cent and the diabetes was obviously uncontrollable. Meat was then given *ad libitum*, with resulting increase followed by decline of glycosuria and further loss of strength. May 28. The dog weighed 9.7 kilos, was too weak to stand, and was killed for autopsy.

*Autopsy.*—The pancreas remnant weighed 5.4 gm. and seemed normal in appearance and consistency. The gross autopsy was otherwise negative except for a large abscess in the right axilla, probably derived from a perforating ulcer at the elbow.

*Microscopic Examination.*—In the tissue removed on May 14 inflammation was limited almost entirely to broad bands of edematous fibrous tissue between lobules, infiltrated with leucocytes and also frequently hemorrhagic, while inside the lobules themselves there was little disturbance. The acini were normal and the islands markedly vacuolated. At autopsy the viscera, including the pancreas, seemed practically normal microscopically. Only slight thickening of trabeculae remained from the previous infiltration. Acini were normal; islands more extensively vacuolated than before and reduced in size and number.

The diabetes was evidently made more severe by inflammation, but even under these circumstances glycosuria was reduced to traces and was on the point of disappearing with 3 days of fasting following

the first operation. By care in diet at this time control of the diabetes could presumably have been achieved, and after subsidence of the inflammation the recovery of a high tolerance could have been expected. Excessive protein diet removed this possibility and, in conjunction with the inflammation, caused a much more rapid course of diabetes and cachexia than usual.

*Results of Prolonged Protein or Protein-Fat Diets.*

Examples were previously given<sup>9</sup> of the existence of an apparent limit of protein tolerance in animals with the proper degree of diabetes; and when such a limit is exceeded, downward progress is natural and inevitable. As part of the protein molecule still remains available for nutrition, the decline of weight and strength is generally slower than on high carbohydrate diets, and life generally continues for several months, as illustrated in numerous former experiments. The above record of Dog B2-56 illustrates downward progress on protein as rapid as is usually seen on carbohydrate-rich diets, and also the facts that differences in the course of experimental diabetes are as marked as in clinical diabetes, and that the characteristic changes in the islands are not dependent on preformed carbohydrate in the diet. A less simple problem of protein feeding is presented under the following conditions.

The question arises whether, when an animal has diabetes, as demonstrated by glycosuria on carbohydrate feeding, but is able to eat protein to satiety without glycosuria, or when the protein ration is kept below the apparent tolerance and the full caloric requirements are supplied by addition of fat, diabetes is permanently avoided or merely delayed. It is essential that the test animals should have no changes in the pancreas tending to lower tolerance, and also that the actual permanency of the latent diabetes should be established and a tendency to spontaneous recovery excluded. With these precautions, it may be assumed that animals with simple resection of a portion of the pancreas are free from constitutional or other inherently progressive processes such as may be imagined in human patients. Tests with prolonged protein feeding in them are of the

<sup>9</sup> Allen,<sup>3</sup> p. 588, Dog 38, p. 777, Dog 154.

highest importance, to decide whether (notwithstanding the high content of potential carbohydrate in protein) the difference between protein and carbohydrate diet is absolute, or whether it is a difference of the kind above mentioned between sugar and starch, in that glycosuria is brought on merely more slowly but just as surely. Even with strict quantitative limitation of protein, the question may be stated on a broader basis with regard to the possibility of an impairment affecting the total metabolism in diabetes; namely, whether a diabetic organism can live out its full normal term of life at a full normal level of weight and metabolism by simple limitation of carbohydrate (preformed or from protein) in the diet, or whether the burden of general metabolism will suffice to wear out the weakened function so that an ultimate outbreak of frank diabetic symptoms will result. Such tests upon suitably chosen animals will contribute much toward the question of spontaneous downward progress in human diabetes and the efficacy of the classical treatment based on the idea of restriction of carbohydrate alone. Dogs are specially suited for such experiments, because of their relative insusceptibility to acidosis and other disturbances on pure protein or protein-fat diets.

The first dogs received upon beginning the investigation were set apart for these prolonged experiments. It was inevitable that deaths from distemper, rabies, and other accidents should spoil years of work in some cases, but by starting with a sufficient series of animals and substituting others as needed, some instructive long observations were obtained, of which the following four are the best examples.

*Dog B2-00.*<sup>10</sup>—Female; mongrel with some bull-terrier blood; brindle; age 3 years; good condition; weight 14 kilos. This dog was received on Oct. 25, 1913, and was subjected to five operations for removal of successive fractions of the pancreas, with periods of many months between so as to allow for any possible compensation by hypertrophy or alteration of assimilation from any cause, and with repeated tests of the carbohydrate tolerance at all stages. Tolerance was maintained for bread and soup with as much as 200 gm. of glucose up to Dec. 16, 1916, when the removal of only 0.1 gm. of pancreatic tissue brought on diabetes, so that bread and soup feeding alone sufficed for slight glycosuria. The dog

---

<sup>10</sup> See photograph at end of Paper 5 (*J. Exp. Med.*, 1920, **xxi**, 587).

passed through two pregnancies in quick succession, the second one terminating on July 16, 1917. The observations up to this point will be described in detail later. Throughout this time she was given 1 kilo of beef lung daily, but was not required to eat all of it; glycosuria was absent except in occasional tolerance tests.

The freedom from glycosuria continuing, the following plasma sugar tests were performed.<sup>11</sup> The time of feeding was between 9 and 10 a.m. daily. July 26. Body weight 13.2 kilos. Dog unwell. Plasma sugar before feeding 0.055 per cent; 2 p.m., 0.099 per cent; 5 p.m., 0.122 per cent; 8.30 p.m., 0.081 per cent. Nov. 27. Weight 14 kilos. 2 p.m. Plasma sugar 0.123 per cent. Dec. 3. Weight 14.5 kilos. Plasma sugar before feeding 0.067 per cent; 5 p.m., 0.108 per cent. Dec. 27. Weight 12.2 kilos. Plasma sugar before feeding 0.128 per cent; 2 p.m., 0.151 per cent; 5 p.m., 0.128 per cent.

TABLE III.

## Dog B2-00.

Time.	Aug. 9, 1917.		Oct. 5, 1917.		Nov. 22, 1917.		Dec. 18, 1917.	
	Plasma sugar. per cent	Urine sugar. gm.	Plasma sugar. per cent	Urine sugar. gm.	Plasma sugar. per cent	Urine sugar. gm.	Plasma sugar. per cent	Urine sugar. gm.
Before feeding.....	0.081	0	0.109	0	0.109	0	0.141	0
2 hrs. after " .....	0.164	Very faint.	0.159	0	0.204	Faint.	0.400	3.55
4 " " " .....	0.109	Faint.	0.145	Faint.	0.238	0.14	0.384	2.10
6 " " " .....	0.135	"	0.152	0.14	0.125	0.11	0.357	3.95

Four tolerance tests also indicated a decline of tolerance during the above period of high protein diet. These tests consisted in giving, on the 4 days mentioned, an identical test diet of 200 gm. of bread, 150 gm. of glucose, and 100 gm. of beef lung, and determining the blood and urine sugars at the intervals shown in Table III.

Jan. 2, 1918. Diet changed to 400 gm. of lung and 50 gm. of suet. Jan. 15. Weight 12 kilos. Plasma sugar before feeding 0.139 per cent; 5 p.m., 0.161 per cent. Jan. 17. Diet changed to bones only. Jan. 21, 2 p.m. Plasma sugar 0.164 per cent. Jan. 28. Weight 11.1 kilos. Plasma sugar before feeding 0.133 per cent. Diet changed to 100 gm. of lung and 100 gm. of suet. Feb. 6, 2 p.m. Plasma sugar 0.155 per cent. Feb. 11. Plasma sugar before feeding 0.112 per cent; 2 p.m., 0.123 per cent. Feb. 14. Diet increased to 300 gm. of lung and 100 gm. of suet. Mar. 14. Diet increased to 500 gm. of lung and 100 gm. of suet.

<sup>11</sup> The methods of Lewis and Benedict (Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61), and of Benedict (Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203) have been used for all blood sugar analyses.

On protein diet from July 26, 1917, to January 15, 1918, the dog showed downward progress as evidenced by the increasing hyperglycemia and the tolerance tests. To exclude the possibility that such a hyperglycemic tendency might be due to something inherent in the dog, undernutrition was instituted on January 17 with a diet of nothing but fresh bones, and thereafter the quantity of protein was restricted, the maximum of 500 gm. of lung being attained by March 14. Hyperglycemia which is slow in onset, due to prolonged excess in protein or fat, is also slow in subsiding; but with the decline in body weight the blood sugar came gradually to the normal level. The last mentioned diet and the freedom from glycosuria continued to August, 1918.

*Dog B2-01.*<sup>10</sup>—Female; bull and fox-terrier mongrel; white with brown markings; age 2 years; good condition; weight 14 kilos. Received Oct. 25, 1913, and used at first like Dog B2-00 for removal of successive portions of pancreas.<sup>12</sup> The removal of 0.8 gm. of tissue in the final operation on Aug. 31, 1916, made the animal potentially diabetic.<sup>13</sup> The tolerance was at first so high that bread diet with 300 gm. of glucose caused no glycosuria. It declined till glycosuria resulted from bread alone, then fluctuated according to the body weight, but on the whole fell in consequence of prolonged slight overfeeding, so that by June 30, 1917, there was hyperglycemia when the dog was fat on a diet of 400 gm. of lung and 200 gm. of suet. The weight was kept low and the tolerance high during the remainder of that year. In 1918 the dog was allowed to gain weight, on a diet which after Feb. 13 was 400 gm. of lung and 100 gm. of suet. The plasma sugar was normal (0.089 per cent) before feeding in the last analysis on Feb. 4.

*Dog D4-52.*<sup>10</sup>—Female; mongrel; yellow and white; age 3 years; good condition; weight 12 kilos. Nov. 24, 1916. Received with pups, which she reared. June 27, 1917. Removal of pancreatic tissue weighing 22.9 gm.; remnant about main duct estimated at 3.4 gm. ( $\frac{1}{4}$ ). Glycosuria at first was heavy on feeding of bread and soup with glucose up to 200 gm., but soon ceased. July 12. 0.35 gm. of additional pancreatic tissue was removed, and the same experience with feeding was repeated. July 20. 0.2 gm. of additional pancreatic tissue was removed, and again the glycosuria from bread and 200 gm. of glucose was transitory. Aug. 3. A final fragment of 0.3 gm. of pancreatic tissue was removed. Glycosuria was absent on bread diet until Aug. 8, when the addition of 200 gm. of glucose made it heavy. On this glucose mixture the sugar excretion gradually diminished and ceased on Aug. 13.

<sup>12</sup> These experiments will be described later.

<sup>13</sup> The subsequent feeding tests will be described later.

After Aug. 21, glucose was omitted and the diet was plain bread and soup. Slight glycosuria appeared on Oct. 10, and the diet was therefore changed to 500 gm. of beef lung and 100 gm. of suet. The same diet was continued regularly, though frequently the dog left considerable of it uneaten. By June, 1918, the weight had risen to a maximum of 12.6 kilos. Tests were performed at intervals (Table IV).

TABLE IV.

*Dog D4-52.*

Feeding of 500 gm. of lung, 100 gm. of suet, and 100 gm. of bread.

Date.	Weight.	Plasma sugar.			Urine volume.	Glycosuria.
		Before feeding per cent	3 hrs after feeding. per cent	6 hrs. after feeding. per cent		
1917	kg.	per cent	per cent	per cent	cc.	per cent
Mar. 1	11.7	0.110	0.122	0.109	420	0
Oct. 25	11.6				580	2.44
Nov. 14	11.7	0.092	0.294	0.246	410	1.1

TABLE V.

*Dog D4-52.*

Feeding of 300 gm. of bread and 200 gm. of glucose.

Date.	Weight.	Plasma sugar.				Glycosuria (24 hrs.). gm.
		Before feeding. per cent	2½ hrs. after feeding. per cent	4 hrs. after feeding. per cent	6½ hrs. after feeding. per cent	
1917	kg.	per cent	per cent	per cent	per cent	gm.
Aug. 8	11.5	0.149		0.312	0.092	6.75
" 15	11.6	0.101		0.172	0.161	0
Oct. 5	11.5	0.208	0.334	0.384	0.416	18.0

The tests mentioned above merely compare the diabetic condition on October 25 and November 14 with the normal state on the preceding March 1. As far as can be judged from glycosuria, there was gain rather than loss of tolerance from October 25 to November 14, though such differences may be accidental.

From the tests in Table V, it is seen that on August 8 (5 days after the last pancreas operation) the above glucose mixture caused hyperglycemia and glycosuria, which were brief, both being ended within the 6½ hour experimental period. By August 15, the bread and glucose

diet having been continued in the interval, tolerance had apparently been gained, so that glycosuria no longer resulted; but the curve of hyperglycemia, though lower, was longer. Delayed absorption is one possible factor here. The dog tired of glucose before diabetes was produced, so that after August it had to be discontinued, as already stated. On plain bread diet thereafter glycosuria was absent, but hyperglycemia was evidently present and tolerance was lost markedly. This was shown in the tolerance test of October 5, in which the blood sugar was high at the outset and ran a prolonged high course. Of the 18 gm. output, only 7.9 gm. were excreted during the  $6\frac{1}{2}$  hour period, and the rest over night. A fast day was given on October 6, to allow recovery from the test. Nevertheless, glycosuria on plain bread and soup feeding began on October 10 as stated. This experiment is another illustration of downward progress on starch diet, and also of downward progress with hyperglycemia without glycosuria. A recuperative effort on the part of the pancreas remnant is manifest, but it was overcome by the excessive feeding.

#### *Intravenous Glucose Tests.*

The intravenous injections were given discontinuously, in a manner described in detail in a later paper. The dosage was constant, on the assumed normal weight of 12 kilos, without regard to the changes in actual weight. This meant the injection of 30 cc. of 10 per cent glucose solution every 15 minutes, in order to give 1 gm. per kilo per hour (Table VI). Feeding was omitted on each injection day and the day following. Excitement and other known causes of disturbance were avoided.

The experiment on August 6, 3 days after the last pancreas operation, seems to show the same characteristics as the above described feeding test on August 8; namely, beginning with an existing hyperglycemia, the blood and urine sugars rose quickly to high levels, but tended to fall toward the close, the relatively low percentages of the 7th and 8th hours being particularly striking.

The observations of November 19 and February 19 show the existence of hyperglycemia on prolonged carbohydrate-free diet, and downward progress of the diabetes notwithstanding absence of glycosuria.

TABLE VI.

Dog D4-52.

Intravenous glucose injections, 1 gm. per kilo per hour in 10 per cent solution (four injections per hour).

Time.	Plasma sugar.	Urine.	
		Volume.	Glucose.
Aug. 6, 1917. Weight 10.75 kilos.			
Befc.e injection.....	per cent 0.218	cc. 16	per cent 0
At end of 1st hr.....	0.500	16	2.64
" " 2nd "	0.715	16	5.08
" " 3rd "	0.475	54	2.84
" " 4th "	0.590	52	1.54
" " 5th "	0.415	76	0.74
" " 6th "	0.270	78	1.81
" " 7th "	0.202	94	1.45
" " 8th "	0.125	82	0.82
1 hr. after injection.....	0.128	53	0.19
2 hrs. " " .....	0.130	12	Faint.
3 " " .....	0.133	29	0
Glucose excreted.....			7.8 gm.
Nov. 19, 1917. Weight 11.7 kilos.			
Before injection.....	0.169		0
At end of 1st hr.....	0.555	36	1.93
" " 2nd "	0.555	68	3.08
" " 3rd "	0.435	82	1.49
" " 4th "	0.370	132	0.79
" " 5th "	0.370	98	0.39
" " 6th "	0.356	126	0.28
" " 7th "	0.370	127	0.55
" " 8th "	0.370	105	0.39
" " 9th "	0.384	105	0.36
" " 10th "	0.322	101	0.42
1 hr. after injection.....	0.156	45	Faint.
2 hrs. " " .....	0.147	10	0
Glucose excreted.....			7.7 gm.
Feb. 19, 1918. Weight 12 kilos.			
Before injection.....	0.145		0
At end of 1st hr.....	0.417	20	4.77
" " 2nd "	0.476	55	5.13
" " 3rd "	0.500	114	3.39
" " 4th "	0.525	109	3.45
" " 5th "	0.500	102	3.40
" " 6th "	0.475	140	2.78
" " 7th "	0.455	90	4.35
" " 8th "	0.384	87	3.23
1 hr. after injection.....	0.294	43	0.74
2 hrs. " " .....	0.170	19	Very faint.
Glucose excreted.....			25.7 gm.

*Dog D4-69.*—Male; black collie mongrel; age 3 years; good condition; weight 15.5 kilos. Jan. 5, 1917. Received. The removal of a kidney on this date was presumably without effect on the production of diabetes. Successive portions of pancreatic tissue were removed on Jan. 23, Feb. 23, Mar. 28, Apr. 19, May 8, June 1, June 27, and July 20. Tests of the tolerance were made between all the above operations, and diets of bread and soup with as high as 400 gm. of glucose, though causing more and more glycosuria, failed to maintain it permanently. After the last operation, which involved the removal of only 0.5 gm. of tissue, glycosuria was absent on bread and soup diet, but heavy at first with the addition of 100 gm. of glucose. As it tended to diminish, the glucose was increased on Aug. 8 to 200 gm., but again the heavy glycosuria diminished, became intermittent, and ceased. No glycosuria occurred after Aug. 15. Aug. 21. Glucose

TABLE VII.

*Dog D4-69.*

Feeding of bread and soup and 200 gm. of glucose.

Date.	Plasma sugar.			Glycosuria (24 hrs.).
	Before feeding. per cent	3½ hrs after feeding. per cent	5½ hrs after feeding. per cent	
1917				
Aug. 8	0 133	0 344	0 310	4.5
" 9	0 116	0 238	0 264	Trace.
" 15	0.110	0 217	0 212	0
Oct. 5	0.200	0 525	0 435	24 8

was discontinued, and the diet thereafter was plain bread and soup. The weight had risen by Oct. 5 to 17.6 kilos. Tolerance tests were performed during this period (Table VII).

Though 2 fast days were imposed following Oct. 5, the former sugar freedom on bread diet was no longer possible, the glycosuria in repeated attempts being persistent and increasing. Therefore, beginning Oct. 17, the diet was 100 gm. of suet and quantities of lung varying from 400 to 1,000 gm. Oct. 25 to 30. A diet of 500 gm. of lung and 100 to 150 gm. of bread caused continuous glycosuria. After this the diet was 500 gm. of lung and 100 gm. of suet, with continuous absence of glycosuria except on a few test days.

Nov. 14. Addition of 100 gm. of bread to the diet caused glycosuria of 0.3 per cent in 350 cc. of 24 hour urine. The plasma sugar before feeding was 0.127 per cent; 3 hours after feeding, 0.244 per cent; 6 hours after feeding, 0.138 per cent.

Intravenous tolerance tests were performed as shown in Table VIII.

Experiments like those on Dogs B2-00, B2-01, D4-52, and D4-69, with their requirements of prolonged care in diets, daily urinalyses, and attention to numerous details, are full of difficulties. These four dogs were the survivors of a series in which the attempt was made to reproduce the conditions of human patients as closely as possible. The above records extend to the summer of 1918, when the writer entered military service, and the dogs were left on the

TABLE VIII.

*Dog D4-69.*

Intravenous injection of 1.3 gm. per kilo per hour of a 10 per cent solution of glucose.

Time.	Aug. 6, 1917. (Weight 16.2 kilos.)			Dec. 4, 1917. (Weight 14.2 kilos.)			Feb. 19, 1918. (Weight 15.5 kilos.)		
	Plas- ma sugar.  per cent	Urine.		Plas- ma sugar.  per cent	Urine.		Plas- ma sugar.  per cent	Urine.	
		Vol- ume.	Glucose.		Vol- ume.	Glucose.		Vol- ume.	Glucose.
Before injection.	0.106	10	0	0.104	0	0.154	32	0	
After 1st hr.	0.278	20	1.56	0.715	100	2.84	0.625	46	4.83
" 2nd "	0.294	30	2.87	0.990	144	4.45	0.800	214	4.00
" 3rd "	0.312	64	1.71	0.990	222	3.65	1.000	252	4.35
" 4th "	0.270	106	0.76	1.000	300	2.80	1.180	274	3.70
" 5th "	0.218	110	0.42	0.844	220	2.93	1.110	281	4.25
" 6th "	0.204	76	0.53	0.844	265	2.43	1.050	270	4.55
" 7th "	0.183	128	0.51	0.625	222	2.71	1.000	222	4.55
" 8th "				0.715	216	3.82	0.950	278	5.13
1 hr. after last injection.	0.082	90	Trace.	0.370	60	3.60	0.525	78	5.88
2 hrs. " " "	0.098	82	0	0.217	66	0.49	0.322	10	5.13
Glucose excreted.....			4.5 gm.			55.2 gm.			80.4 gm.

Both the feeding and the intravenous tests indicated gradual loss of tolerance in the absence of glycosuria, first on starch and later on protein-fat diet. The sugar curves of Feb. 19, 1918, were not only the highest and most prolonged, but also with the initial figure of 0.154 per cent indicated that hyperglycemia was now constant on the regular diet of lung and suet. In the final blood taken at the height of digestion at 2.30 p.m. on Mar. 20, 1918, the plasma sugar was 0.256 per cent, still without glycosuria. The weight was gradually falling, and was 14.75 kilos at this time.

diets stated under the care of the animal attendants. They were seen once during the winter, sufficiently to learn that all four were free from glycosuria; three of them were vigorous and fat, but Dog D4-69 was thin and in rather poor condition. In the late summer of 1919, two of the dogs were found to have hyperglycemia and glycosuria, and were therefore transferred from The Rockefeller Institute to the writer's clinic. The two which were sugar-free were cared for at the farm of the Institute in New Jersey.

The animal in worst condition was Dog D4-69, which was thin, with hopeless hyperglycemia and glycosuria, and which died of diabetes on October 10, 1919. Dog B2-01 was strong and fat at a weight of 14.8 kilos, but showed heavy sugar and acetone reactions. Glycosuria was at first abolished by fasting, but returned, owing to persistence of hyperglycemia when attempts were made to feed. Rigorous undernutrition at this time might have proved successful, but the mistaken laxness, due to the deceptive strength and fatness of the animal, soon ended in a hopeless condition, which was uncontrollable by fasting, so that death occurred November 15, 1919. The record of the terminal period is given in the next paper. The other two dogs were in excellent condition when visited at the farm on November 3, 1919. Plasma sugar analyses on that day were as follows: Dog B2-00, before feeding, 0.092 per cent; 4 hours after feeding, 0.136 per cent; Dog D4-52, before feeding, 0.093 per cent; 4 hours after feeding, 0.123 per cent.

It will be seen that the experiment consisted in taking four dogs with different grades of diabetes, which had been produced by sufficiently long experimental procedures to rule out accidental influences as far as possible, and had been tested in various feeding and injection experiments, and then placing them on nearly identical diets on which they were free from glycosuria for extended periods. The ultimate outcome could in the main have been predicted from the preliminary observations.

Dog D4-69 had continuous hyperglycemia in the spring of 1918, and in the absence of extraneous interfering factors such a condition in dogs always leads to manifest and fatal diabetes. Loss of weight may have been due to the latent diabetes or to indigestion or other causes; it evidently postponed the outbreak of active dia-

betes longer than usual. Dog B2-01 exhibited hyperglycemia and lowered tolerance when obese in the summer of 1917, and it was therefore to be expected that a similar result would follow when she became fat on the same protein ration in 1918-19. These records illustrate that in dogs, as in human patients, a true recuperative power is limited to the earlier stages of diabetes, and when diabetes has lasted many months or years the assimilation may undergo apparent fluctuations according to the diet and weight but is not capable of any great restoration in an absolute sense. The final purpose was, after the expected onset of diabetes, to check this as before by fasting and reduced diet, and then prove that the animals could be kept indefinitely in the undernourished condition free from diabetes or downward progress, on the same protein ration; in other words, to show the influence of dietary fat and body weight in causing downward progress. This fact was established by other experiments,<sup>12</sup> but the opportunity of testing the permanency of such arrest of diabetes and the feasibility of protecting the assimilation by undernutrition extending over many years or the full lifetime of the dogs was lost.

Dogs B2-00 and D4-52 had milder diabetes. Both showed downward progress on excessive diets of carbohydrate or protein, and both illustrated the fact, often observed in human patients, that when mild diabetes is checked by a diet within the actual tolerance, hyperglycemia may persist for a long time but gradually subsides. The question was what would happen to such animals if they were allowed to live indefinitely on a diet which seemed to be within the tolerance. The result could not be predicted in advance, and the outcome to date establishes the following conclusions.

1. No inherent downward progress is perceptible. Dog B2-00 has been kept for 6 years, and has been demonstrably diabetic for 3 years. The downward progress observed in other animals, and also in these animals on excessive diets, is purely the result of food injury; in other words, to functional overstimulation of the pancreas as an endocrine organ.

2. The benefit of the classical treatment of diabetes is confirmed. With the susceptibility of both these animals to injury from excess of either carbohydrate or protein demonstrated, it is evident that

this injury was checked when carbohydrate was omitted, protein restricted, and a full caloric diet made up by the use of fat. Fat is evidently less injurious than carbohydrate or protein, and its harmfulness is chiefly noticeable in the more severe grades of diabetes. It is safer for these animals to be obese on a fat diet than to eat carbohydrate or a carbohydrate-forming food such as protein. By inference, fat is not a direct source of carbohydrate.

3. The future outcome in these two animals holds several possibilities. (a) One question concerns the degree and permanence of their assimilative power. Other experiments justify the assumption that by undernutrition their tolerance could be greatly raised, so that they might take considerable protein and carbohydrate, and by increased obesity the tolerance could be further lowered, probably to the point of glycosuria on their present protein ration. The question is whether their tolerance at their present weight is high enough for permanent assimilation of the limited carbohydrate derivable from their present diet; whether the difference between fat and protein is absolute or merely a matter of time, like the differences between glucose and starch or between starch and protein; and therefore whether diabetes is primarily a deficiency of the total metabolism or of carbohydrate metabolism alone. (b) The plasma sugars under present conditions are not absolutely normal. Particularly in Dog B2-00 the figure of 0.136 per cent during digestion of protein is above normal, but yet is within the limits permitted in numerous diabetic patients. There is a question whether this slight and temporary overload of each day can be borne indefinitely by the pancreas, or whether it is the first small sign of a breaking strain. (c) In addition to the simple prolonged functional wear and tear, other influences such as age enter in, and it is of interest to know whether senility will bring an onset of diabetes, as it seems to do in so many human patients. If necessary, the direction of progress in the animals can be judged at any time by tolerance tests, which were instituted in the first place in order to permit of such comparisons, but it seems better to avoid this source of possible injury. As these dogs bear such a close resemblance to mildly diabetic patients kept sugar-free by restriction only of sources of carbohydrate, it seems most valuable to learn whether they can remain fat and lazy indefinitely.

with impunity, or whether even the mildest diabetes will ultimately undergo aggravation from luxus diets.<sup>14</sup>

#### CONCLUSIONS.

No specific differences were observed between the glycosuric effects of different kinds of protein. Other incidental observations were noted in connection with the records of individual animals. General conclusions are deferred to the close of the series.

<sup>14</sup> Both these dogs recently died and were autopsied. Dog D4-52 had a large calculus in the bladder but otherwise appeared to be in excellent condition. No cause of death was found. Dog B2-00 was more closely observed; there was a history of refusal of food, and drinking and vomiting of water. The urine in the bladder was heavy with sugar; the subcutaneous tissue and peritoneum contained abundant fat, and the liver was intensely fatty. It is therefore certain that the death of this animal was due to acidosis. The same possibility exists for Dog D4-52 but is unproved. The results indicate that luxus diets lead to a fatal termination in diabetes



## EXPERIMENTAL STUDIES ON DIABETES.

### SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

#### 4. CONTROL OF EXPERIMENTAL DIABETES BY FASTING AND TOTAL DIETARY RESTRICTION.

By FREDERICK M. ALLEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 8, 1919.)

In a previous publication the writer reported the results of attempts to produce a satisfactory experimental reproduction of clinical diabetes and a study of its nature, which was made to obtain some clue leading to its therapeutic control.<sup>1</sup> This investigation was carried to a point where it was believed, as stated in the preface, that the possession of the necessary working model of the disease had made the cure of diabetes a feasible laboratory problem. Out of numerous lines of therapeutic approach suggested from the literature, speculation, or experiment, two seemed worth following but had to be left unexplored. One of these consisted in attempts to stimulate or strengthen the pancreatic function in some direct manner, and it is still hoped to proceed in this direction at some later date. For certain reasons it was desirable to follow the other path first.

The phenomenon which suggested this latter research was the prevention or cessation of diabetes with ligation of the pancreatic duct.<sup>2</sup> It was at first suspected that this was due to improvement of the internal pancreatic function, through stoppage of the external secretory activity or even through structural changes involving increase of island tissue. When the work was finished, there was leisure for the following comparison with other observations. In Dog 55<sup>3</sup> diabetes ceased with the onset of distemper and the attendant loss of appetite and weight. In Dog 57, fasting for a week prior to operation seemed to prevent the onset of glycosuria. Likewise peritonitis and other infections often hindered the occurrence of glycosuria, though in some instances diabetes appeared as usual, notwithstanding the presence of peritonitis. Several other dogs<sup>4</sup> showed absence or cessation of glycosuria in consequence of illness, fasting, and loss of weight.

<sup>1</sup> Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913.

<sup>2</sup> Allen,<sup>1</sup> Chapter XXII.

<sup>3</sup> Allen,<sup>1</sup> p. 493.

<sup>4</sup> Allen,<sup>1</sup> pp. 771-772.

Also in an animal with fairly severe and advanced diabetes,<sup>5</sup> glycosuria was twice stopped by fasting, and downward progress in consequence of the overfeeding during the interval was demonstrated by the lower tolerance for protein at the close of the second as compared with the first fast. There are reports in the literature of the apparent cessation of diabetes with an intercurrent cachectic disorder,<sup>6</sup> and also of the benefit of the occasional therapeutic fast days introduced by Bouchardat, Cantani, and Naunyn. In addition, shortly before the beginning of the new research, Joslin emphasized the subsidence of diabetic symptoms in a severe case after the onset of tuberculosis with emaciation.<sup>7</sup> It thus seemed possible that the cessation of glycosuria from ligation of the pancreatic duct resulted from impaired food absorption and undernutrition. Homans<sup>8</sup> confirmed the phenomenon itself and demonstrated this as the true explanation by direct experiments.

The first opportunity was taken to try reduction of weight and total metabolism for the treatment of diabetes. The first dogs were chosen for the longest experiments, as explained in the preceding paper. (It so happened that Dog B2-05 furnished another illustration of the cessation of glycosuria with distemper.) The tests consisted in taking dogs, generally with much smaller pancreas remnants than those with which downward progress and death were found on full diets in the preceding paper, and determining to what extent active symptoms and impairment of assimilation could be prevented by fasting and total dietary restriction. Many examples are available and are mentioned incidentally in other papers, because the method was used as a routine for controlling diabetes. As the longest records are the most instructive for the present purpose, three experiments of 1 to  $1\frac{1}{4}$  years in duration are here summarized as successful cases. Before proceeding to these, brief consideration may be given to the status of the so called "hunger glycosuria" in this connection.

*Dog B2-52.*—Female; bull-terrier mongrel; brindle; excellent condition; weight 12.8 kilos. Apr. 24, 1914. Removal of pancreatic tissue weighing 20.4 gm. Remnant about main duct estimated at 3.1 gm. (slightly over  $\frac{1}{4}$ ). Glycosuria

<sup>5</sup> Allen,<sup>1</sup> Dog 64; pp. 354-361, 480.

<sup>6</sup> Allen,<sup>1</sup> p. 800 ff.

<sup>7</sup> Benedict, F. G., and Joslin, E. P., *Carnegie Inst. Washington, Pub. No. 136*, 1910. Joslin, E. P., *Treatment of diabetes mellitus*, Philadelphia and New York, 2nd edition, 1917; 409.

<sup>8</sup> Homans, J., *J. Med. Research*, 1915, xxxiii, 1.

was absent till bread and soup were fed on Apr. 30. It was maintained till May 7 by the addition of as much glucose as possible, but then ceased largely because of the dog's distaste for sugar. May 12 to 15. Fasting was imposed. The feeding of bread and soup with 50 to 75 gm. of glucose daily then caused glycosuria of 0.7 per cent on the 1st day, but none thereafter, and a second operation was necessary to make the dog diabetic.

This dog is representative of several tested in relation to hunger glycosuria, some of which were previously mentioned.<sup>9</sup> The point involved is the seeming conflict between the use of fasting and under-nutrition for raising tolerance, and the experience of Hofmeister and others concerning the tendency to glycosuria created by starvation and malnutrition. When a dog on the border-line of diabetes refuses to eat enough to keep up glycosuria and break down tolerance, it might seem a promising plan to fast for a number of days and then feed bread and glucose, with the double idea that the glycosuric tendency would be increased by the fast, and also that the animal would eat more. Hofmeister's work is confirmed in these dogs much more strikingly than in normal dogs, and heavy glycosuria often occurs on feeding bread either alone or with sugar. The same phenomenon has frequently been witnessed in dogs changed suddenly from a protein or fat diet to a carbohydrate diet. But no matter how heavy the glycosuria, it is transitory just as in Hofmeister's normal dogs, and it has never been possible to produce diabetes thus in any animal which was non-diabetic on the same diet before the fast. The phenomenon seems to illustrate some state of unpreparedness of the body for the unaccustomed carbohydrate flood, but it does not represent any true diabetic tendency or any exception to the general rule that the pancreatic function is strengthened by fasting.

*Dog B2-25.*<sup>10</sup>—Male; mongrel; tall, rough haired, brown and white; age 3 years; moderately well nourished; weight 18.2 kilos. Dec. 16, 1913. Removal of pancreatic tissue weighing 30.1 gm. Remnant about main duct estimated at 2.6 gm. ( $\frac{1}{4}$ — $\frac{1}{2}$ ). The further record is contained in Table I. Dec. 3, 1914. In excellent health and strength; accidental death.

*Autopsy.*—Negative grossly and microscopically. Pancreas remnant, fully normal in appearance and consistency, weighed 8.25 gm. Microscopically, acini normal; islands small and scarce, free from vacuolation.

<sup>9</sup> Allen,<sup>1</sup> pp. 586-587. Cf. Paper 3, p. 557; also Dog B2-51, p. 578.

<sup>10</sup> See photograph at end of Paper 5 (*J. Exp. Med.*, 1920, xxxi, 587).

The animal lived approximately a year, was most of the time hungry and excessively thin, but vigorous and lively, in contrast to the weakness of diabetes. Tolerance was very low at the outset, corresponding to the small pancreas remnant; but instead of downward progress there was a very great gain in assimilation. The clinical absence of diabetes was corroborated by the intact state of the islands.

TABLE I.  
*Dog B2-25.*

Date.	Body weight.	Diet.	Urine.	
			Volume. cc.	Glucose. per cent
1913				
Dec. 17	18	Fasting.	1,025	0
" 18		200 gm. of meat.	1,500	1.0
" 19		Fasting.	1,700	0.6
" 20		"	875	Faint. 0
" 21-26		"		
To May, 1914	Down to 11.	From 30 gm. of lung and 30 gm. of suet to 120 gm. of lung and 120 gm. of suet.		Traces of glycosuria at first on the higher diets; later none.
" Dec., 1914	Increase to 17.	Pancreas or lung increasing as described in pre- ceding paper,* up to 1,400 gm. daily.		Glycosuria absent till Nov. 25-27; then 4.1 to 8.2 gm.; stopped by a fast day.

\* Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 556.

*Dog B2-51.*—Male; mongrel; yellow and white; age 1½ years; moderately well nourished; weight 9.75 kilos. Mar. 31, 1914. Removal of pancreatic tissue weighing 20.1 gm. Remnant about main duct estimated at 2.8 gm. (†). Beginning Apr. 2, a diet of 200 cc. of milk daily caused glycosuria as high as 2.6 per cent, and on Apr. 5 the change to bread and soup diet resulted in glycosuria of 5 per cent; but this gradually subsided and could only be maintained by the addition of 50 gm. of glucose, which was as much as the dog would take on account of a decided distaste for sugar. With increasing indigestion and loss of weight, glycosuria ceased on Apr. 20.

Apr. 21. Body weight 8 kilos; opening the abdomen revealed obvious marked hypertrophy of the pancreas remnant, from which 1.5 gm. of tissue were removed. Glycosuria was then absent on meat diet. Apr. 26. An abrupt change to bread and soup produced glycosuria as usual for 1 day; it then remained absent even with the addition of 50 gm. of glucose on Apr. 30.

May 1. An additional 1.8 gm. of pancreatic tissue was removed, the body weight being 8.1 kilos. A slight glycosuria ensued on that day, then was absent on beef lung, and after May 7 on bread and soup with 50 gm. of glucose. The emaciated and cachectic condition was a sufficient explanation; when the full diet was forced it was probably largely lost through diarrhea, and on May 12 the weight was down to 6.4 kilos. Accordingly meat diet *ad libitum* was then resumed, with gradual

TABLE II.  
Dog B2-51.

Date.	Body weight.	Diet.	Glyco-suria. per cent
<i>1914</i>	<i>kg.</i>		
July 1-9	8.7-7.6	100 gm. of lung.	0
" 10-22	7.6-7.0	200 " " "	0
" 23-30	6.9-6.75	250 " " "	0
" 31-Aug. 9	6.7-6.6	300 " " "	0
Aug. 10-14	6.6-6.5	325 " " "	0
" 15-19	6.5-6.7	100 " " " 100 gm. of suet.	0
" 20-26	6.8-6.5	100 " " " 100 " " "	0
" 27-Oct. 6	6.6-7.2	100 " " " 75 " " "	0
Oct. 7-15	7.2	100 " " " 50 " " "	0
" 16-Nov. 5	7.2-6.8	100 " " " 30 " " "	0
Nov. 6-Dec. 1	6.9-7.2	100 " " " 30 " " " 50 gm. of lard.	0
Dec. 2-13	7.2	150 " " " 30 " " "	0
" 14-21	7.2-6.6	150 " " "	0
" 22-Jan. 3,			
1915	6.5	150 " " " 50 " " lard.	0
<i>1915</i>			
Jan. 4-Feb. 17	6.6-8.0	150 " " " 100 " " "	0
Feb. 18-Mar. 1	8.0-8.1	200 " " " 50 " " "	0
Mar. 2-18	8.2-8.9	150 " " " 100 " " "	0
" 19	8.8	400 " " "	Trace.
" 20	8.8	400 " " "	0.16
" 21	8.6	Fasting.	0.36
" 22	8.4	100 gm. of lung.	0.25
" 23	8.4	100 " " "	0

gain in weight and health. By June 24, the weight had risen to 9.3 kilos. Glycosuria, which had remained absent before, suddenly appeared and continued for 5 days, the highest percentage being 1.2. It was stopped by 2 fast days; the diet and progress after these fast days are shown in Table II. Mar. 26, 1915. Accidental death.

*Autopsy.*—The pancreas remnant, normal in appearance and consistency, weighed 2.75 gm. Islands were rather few and small, but within normal limits.

Occasional cells in them showed slight vacuolation. Liver moderately fatty. Otherwise autopsy negative, including microscopic examination of thyroid, liver, kidneys, adrenals, and hypophysis.

Pancreatic tissue removed by operation Apr. 21, 1914, normal except for slight vacuolation in occasional island cells. Pancreatic tissue removed May 1, 1914, normal except for slight inflammatory reaction in places; no vacuolation in islands.

It is probable that the dog might have been made diabetic after the first operation if the sugar feeding could have been forced as in some other animals. This is suggested by the glycosuria and the anatomic evidence of overtaxed function in the island cells; but at the same time hypertrophy of the remnant and fall of body weight had the opposite influence, and repugnance to carbohydrate, indigestion, and diarrhea closed the attempt. Prior to the second operation, glycosuria was absent, and correspondingly the islands showed no vacuolation. Shortly preceding death there was slight glycosuria on protein diet, and this was paralleled as usual by slight hydropic changes in the islands. This experiment is one illustration that neither the size of the pancreas remnant, inflammatory changes, the length of time since a preceding operation, nor the character of the diet (carbohydrate or protein) govern this change in the islands, but only the presence or absence of active diabetes.

The record from May 1 to July 1, 1914, illustrates absence of diabetes in an animal in an emaciated condition on pure protein diet, and the loss of tolerance and outbreak of active symptoms when weight was gained on this diet.

The subsequent record shows the absence of diabetes on a restricted protein-fat diet, frequently adjusted in minor details to suit the dog's appetite and digestion, but so planned as to keep the body weight generally low. The animal was lively and fairly strong meanwhile. There was no tendency to real recovery, for when the weight was allowed to rise at the end, it was found that 400 gm. of lung sufficed to cause slight glycosuria. The slight vacuolation found in the islands resulted from such periods of overfeeding. The principal point of the experiment was the ability to keep a thin animal with diabetes of this degree of severity free from symptoms, without evidence of downward progress or indications that the condition could not have been continued indefinitely.

*Dog D4-28.*—Male; mongrel; yellow and white; age 2 years; good condition; weight 12 kilos. Sept. 28, 1916. Removal of pancreatic tissue weighing 25.3 gm. Remnant about main duct estimated at 2.3 gm. ( $\frac{1}{12}$ ). As this dog had been born and raised on the Institute farm and was known to have lived all his life on bread and cereals, after operation advantage was taken of the opportunity to test the effects of fasting and fat diets, as will be described in a subsequent paper on acidosis. Neither glycosuria nor acidosis occurred, and the plasma sugar on Oct. 2 was 0.128 per cent, on Oct. 3, 0.092 per cent, and on Oct. 5, 0.101 per cent. After Oct. 5 the dog was kept on rather low protein-fat diets, maintaining a body weight of 10.5 to 10.2 kilos.

Oct. 29. A pan of bread and soup was fed, causing the excretion of 2.39 per cent sugar in 663 cc. of urine.

TABLE III.

*Dog D4-28.*

Date.	Urine.		Remarks.
	Total nitrogen.	Ammonia nitrogen.	
1917	gm.	gm.	
Jan. 28	2.14	0.08	
" 29	3.73	0.19	
" 30	1.34	0.11	
" 31	2.33	0.11	
Feb. 1	1.55	0.10	
" 2	2.98	0.30	Total nitrogen in the feces for the period 4.46
" 3	2.62	0.27	gm.

Oct. 30. A diet was fed of 200 gm. of beef lung, 200 gm. of suet, and 75 gm. of bread, without glycosuria. The plasma sugar before feeding was 0.184 per cent. Taken at 2 hour intervals for 12 hours after feeding it was 0.184, 0.200, 0.222, 0.213, 0.184, and 0.172 per cent. The following morning before feeding it was 0.179 per cent.

Nov. 2. The diet was 200 gm. of lung, 150 gm. of lard, and 100 gm. of bread. The plasma sugar before feeding was 0.143 per cent. Taken at 2 hour intervals for 12 hours thereafter it was 0.166, 0.180, 0.200, 0.208, 0.182, and 0.182 per cent, without glycosuria.

Nov. 3, 10 a.m. Plasma sugar 0.148 per cent. The dog was then fed only 200 gm. of lung. 5 p.m. Plasma sugar 0.143 per cent.

Beginning Nov. 4, on a diet of 200 gm. of lung and 100 gm. of suet, the dog gradually gained weight up to 11.3 kilos on Dec. 4. On that day faint glycosuria appeared, and was absent the next day. On Dec. 6 there was excretion of 0.74 per cent glucose in 124 cc. of urine, on Dec. 7, 0.59 per cent in 136 cc. of urine, and

on Dec. 8, 1.81 percent in 396 cc. of urine, with a trace of acetone. With 1 day of fasting there were only traces of sugar and acetone, and a 2nd fast day cleared up both. Then the feeding of 100 gm. of suet failed to bring back either sugar or acetone. These remained absent thereafter on a diet of 100 gm. of lung and such

TABLE IV.  
Dog D4-28.

Date.	Urine.		Remarks.
	Total nitrogen.	Ammonia nitrogen.	
1917	gm.	gm.	
Apr. 10	1.69	0.10	
" 11	1.88	0.27	
" 12	1.24	0.10	
" 13	2.40	0.19	
" 14	2.46	0.25	
" 15	1.60	0.16	
" 16	1.28	0.12	
" 17	3.32	0.28	
" 18	1.97	0.20	Total nitrogen in the feces for the period
" 19	2.08	0.22	1.69 gm.

TABLE V.  
Dog D4-28.

Date.	Urine. Total nitrogen.	Date.	Urine. Total nitrogen.
1917	gm.	1917	gm.
Oct. 5	1.12	Oct. 13	—
" 6	1.64	" 14	2.18
" 7	2.04	" 15	2.06
" 8	1.78	" 16	1.82
" 9	1.74	" 17	1.60
" 10	2.36	" 18	1.96
" 11	2.18	" 19	3.24
" 12	—	" 20	2.52

small quantities of suet as the dog chose to eat. Dec. 27. Plasma sugar 0.084 per cent, with hemoglobin (Fleischl-Miescher) 114 per cent. Jan. 18, 1917. Plasma sugar 0.083 per cent, with hemoglobin 111 per cent. The weight during this time up to Jan. 27 ranged from 10.4 to 11 kilos.

Jan. 27 to Feb. 3. A metabolism experiment was conducted on a diet of 100 gm. of lung and 100 gm. of suet. The dog was catheterized on these two dates,

but in the interim passed urine spontaneously and regularly. Feces were combined and analyzed in one lot for the entire period. The results are given in Table III.

Beginning Mar. 10, at a body weight of 10.4 kilos, the above diet was increased by 25 gm. of bread. After Mar. 13, this was changed to 50 gm. of lung, 50 gm. of bread, and 100 gm. of suet. Apr. 10 to 19. Urinalyses were performed on the latter diet (Table IV).

The body weight gradually rose from 10 kilos on Mar. 20 to 10.8 kilos on June 30. June 15. The plasma sugar before feeding was 0.081 per cent; 4 hours after feeding, 0.161 per cent. Suet was then omitted; this made the diet only 50 gm. of lung and 50 gm. of bread. The weight was thus reduced by July 17 to 9.75 kilos. After that, 50 gm. of suet were resumed, but were omitted at times as the dog tired of it, so that the body weight was kept between 9.2 and 10 kilos. Oct. 5 to 20. A series of urinalyses, omitting feces, was performed on the usual diet of 50 gm. of lung, 50 gm. of bread, and 50 gm. of suet (Table V).

Nov. 26. The above diet was fed about noon. 3 p.m. Plasma sugar 0.118 per cent. Beginning Nov. 27, bread alone was fed, in increasing quantities, from 150 gm. on the 1st day to 300 gm. on Nov. 30, without glycosuria.

Dec. 1, 12.30 p.m. The dog was fed 300 gm. of bread with 100 gm. of glucose, without glycosuria. The plasma sugar before feeding was 0.13 per cent, and at 4 p.m., 0.216 per cent. Dec. 2. Diet the same. Dec. 3. The glucose was increased to 150 gm., still without glycosuria. Dec. 4. Plain bread and soup mixture was fed. Dec. 5 to 7. 200 gm. of glucose were added daily, with glycosuria never above 0.61 per cent. Dec. 8. The plasma sugar was 0.161 per cent, and the diet was changed to lung *ad libitum*. The body weight at this time was 9 kilos.

Beginning Dec. 14, 100 gm. of suet were added, and on 2 days, *viz.* Dec. 14 and 26, a pan of bread and soup was allowed in addition, without glycosuria. The weight thus rose to 10.8 kilos.

A test with carbohydrate was then performed, in comparison with the one a month previous at a lower weight. Glycosuria was absent on bread diet. On Jan. 3, 1918, when 300 gm. of bread with 100 gm. of glucose were fed to compare with Dec. 1, the plasma sugar before feeding was 0.154 per cent and at 4 p.m., 0.452 per cent, with excretion of 3.8 per cent sugar in 700 cc. of urine for the 24 hours.

Jan. 4. Glycosuria ceased on a diet of 300 gm. of lung and 100 gm. of suet.

Jan. 5. On feeding plain bread and soup there was excretion of 3.45 per cent sugar in 415 cc. of urine.

Thereafter the diet of 50 gm. of lung, 50 gm. of suet, and 50 gm. of bread was resumed. A gradual fall in weight ensued, to 9.75 kilos on Jan. 28, 8.75 kilos on Feb. 21, 8.3 kilos on Mar. 11, and 7.4 kilos on June 11. Glycosuria was constantly absent, and up to June 8 the dog maintained the utmost activity. The urine, as collected under the cage, then became increasingly bloody. The cause

was supposed to be bloody diarrhea, due to deficient diet, as described by Rosenheim. The diet was therefore enriched with an abundance of fresh meat, bones, milk, eggs, and yeast, to supply any missing elements. Nevertheless, the condition became worse. June 12. The animal was found dying and was killed for autopsy. The plasma sugar of the heart's blood was 0.159 per cent, the carbon dioxide capacity 32.8 volumes per cent, without acetone or other evidence of acidosis.

*Autopsy.*—The cause of death was found to be urinary obstruction from a large calculus impacted in the urethra near the bladder, with several smaller stones lodged behind it. More or less urine had been passing up to the day of death, but the bladder and both ureters and kidneys were distended with bloody urine, which had also infiltrated extensively about the neck of the bladder. The intestine was normal throughout and there was no blood in its contents anywhere. The other viscera were negative. The pancreas remnant, normal in appearance and consistency, weighed 3.5 gm.

*Microscopic Examination.*—The kidneys were inflamed and infiltrated with mononuclear cells chiefly in the cortex and polymorphonuclears chiefly in the medulla. The Armanni or Ehrlich vacuolation was also evident in some tubules. The liver was normal and its cells contained no visible fat. The pancreas remnant was entirely normal and free from fibrosis. The acini were regular and well filled with zymogen, and the islands normal in number and size and free from vacuolation. The other organs were normal.

As the experiment was a long one, the opportunity was taken to use it for several purposes simultaneously. Aside from the acidosis observations mentioned, the following points were included. As low protein diets, sometimes in conjunction with undernutrition, are an occasional feature of diabetic treatment, it was desirable to test the feasibility of such a program in the dog. This was especially desirable in regard to any possible doubts still persisting from the work of Munk,<sup>11</sup> Rosenheim,<sup>12</sup> and Zuntz and Magnus-Levy,<sup>13</sup> though it seems generally accepted that the difficulties reported by these investigators were due not to protein deficiency but to other unsuitable features in the diets used. One of these features is considered to be the use of meat powder in Munk's 10 week experiment. Rosenheim noticed decline of appetite and digestion within 8 weeks, perhaps

<sup>11</sup> Munk, I., *Arch. Physiol.*, 1891, 338; abstracted in *Jahresb. Fortschr. Thier-Chem.*, 1892, xxi, 365.

<sup>12</sup> Rosenheim, T., *Arch. Physiol.*, 1891, 341; abstracted in *Jahresb. Fortschr. Thier-Chem.*, 1892, xxi, 365.

<sup>13</sup> Zuntz, N., and Magnus-Levy, A., *Arch. ges. Physiol.*, 1891, xliv, 438.

due to the rice used, and then obtained a fatal result by changing to an excessive fat ration. The dog described above (No. D4-28) had presumably some tendency to impaired digestion created by the removal of  $\frac{1}{2}$  of the pancreas, yet suffered none of the disturbances described by these authors. A little bone-meal daily prevented diarrhea and presumably contributed some mineral salts. From March 14 to November 27, 1917, the dog remained in excellent health and spirits on 50 gm. of beef lung (cooked), 50 gm. of bread, and 50 to 100 gm. of raw beef suet. Weight was gained whenever the calories were sufficient and was lost in normal manner whenever the dog tired of too much suet. After January 6, 1918, there was a gradual and regular loss of weight up to June, on the undernutrition diet of 50 gm. of lung, 50 gm. of bread, and 50 gm. of suet. During these long periods there was no sign of any specific disorder due to the reduction of urinary nitrogen to approximately 2 gm. daily.

During this same time, the animal served as a control for other dogs on high fat diets, as will be described in a later paper.

The influence of body weight upon assimilation was twice observed; namely, the absence of glycosuria on 200 gm. of lung, with fat and sometimes 75 or 100 gm. of bread up to November 4, 1916, at weights below 10.5 kilos, and the occurrence of glycosuria a month later on 200 gm. of lung and 100 gm. of suet at a weight of 11.3 kilos; likewise the lower tolerance of early January, 1918, at a weight of 10.8 kilos, as compared with a month preceding at a weight of 9 kilos. The weight was finally allowed to fall very low, with a view to demonstrating a still higher tolerance, and then ultimately bringing back diabetes by fattening; but this plan could not be carried out because of the accidental death.

This dog was characterized throughout by oliguria and high renal thresholds for sugar. There is a question of the possible existence and injury of renal calculi from the outset, but the appearance of the kidneys did not suggest a very long standing trouble. Similar peculiarities of function have been observed in other dogs under similar conditions, and the conclusions regarding diabetes are supported by blood as well as urine analyses.

The experiment also served as a test of the specific influence of pre-formed carbohydrate as a cause of injury to the assimilation in dia-

betes. An animal was chosen with potentially severe diabetes, produced by removal of  $\frac{1}{2}$  of the pancreas and demonstrated by the occurrence of glycosuria on December 4 to 8, 1916, on a diet of only 200 gm. of lung and 100 gm. of suet, when the body weight had, as stated, been built up to a level which was still subnormal. Under these conditions the dog was undernourished by means of a mixed diet containing an appreciable quantity of carbohydrate, and the experiment was continued long enough to test whether carbohydrate would prove itself a specific poison to the islands of Langerhans. The actual result was a marked gain in tolerance. This is best seen by comparison of the above mentioned glycosuria in December, 1916, on 200 gm. of lung and 100 gm. of suet at a weight of 11.3 kilos, with the tests a year later when 300 gm. of bread could be taken without glycosuria at a weight of 10.8 kilos, and when the glycosuria which was produced by the addition of glucose cleared up (January 4, 1918) on 300 gm. of lung and 100 gm. of suet. It may be concluded that the tolerance created by undernutrition was genuine, and that the feeding of carbohydrate within the limits of this tolerance did not damage the assimilation or the islands of Langerhans.

Apart from the above details, the main point of the experiment was to determine whether downward progress is inevitable in an animal with potentially severe diabetes, so that symptoms must ultimately occur in spite of dietary restriction. This dog was kept for approximately  $1\frac{3}{4}$  years. Besides the gain in tolerance during this time, the intact state of the islands furnished additional evidence that no injurious change was in progress, and the indications are that the same condition of health could have been maintained indefinitely.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1920, Vol. xxxi,  
No. 5, pp. 587-608.]

## EXPERIMENTAL STUDIES ON DIABETES.

### SERIES I. PRODUCTION AND CONTROL OF DIABETES IN THE DOG.

#### 5. VARIOUS FAILURES OF DIETETIC TREATMENT, AND THEIR CAUSES.

By FREDERICK M. ALLEN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATES 65 AND 66.

(Received for publication, December 8, 1919.)

#### *Glycosuria Controllable Only by Fatal Undernutrition, Because of Severity of Diabetes.*

*Dog B2-28.*—Male; mongrel; black and white; age 1 year; slightly thin; weight 11 kilos. Dec. 18, 1913. Removal of pancreatic tissue weighing 32 gm. Remnant about main duct estimated at 2 gm. ( $\frac{1}{7}$ ). Glycosuria of 0.55 per cent followed the operation, but diminished, and by Dec. 23 was absent. Continued attempts were then made to nourish the animal with low protein-fat diets, often subdivided into several meals during the day, but traces of glycosuria recurred each time, requiring further fasting and undernutrition. Feb. 24, 1914. The dog was at the point of death from undernutrition; weight 6.8 kilos; killed for autopsy.

*Autopsy.*—The pancreas remnant, not obviously sclerosed, weighed 1.2 gm.

*Microscopic Examination.*—Portions of the remnant showed interacinar pancreatitis, other portions normal parenchyma. The islands generally were few and small but not to the extent of a positively pathological reduction. Appearances of vacuolation were rare and doubtful, but search through many sections revealed a very few distinct examples.

*Dog B2-29.*—Male; mongrel; white; age 1½ years; good condition; weight 10.6 kilos. Dec. 22, 1913. Removal of pancreatic tissue weighing 27.7 gm. Remnant about main duct estimated at 2.4 gm. ( $\frac{1}{2}-\frac{1}{8}$ ). Only faint glycosuria followed the operation. The subsequent undernutrition was more rigid than in Dog B2-28, in the sense that no more than a trace of glycosuria on a single day was permitted at any time. The same inability to develop a tolerance for any living diet was encountered, and by Apr. 7, 1914 the weight had declined to 5.9 kilos. On that day the dog was killed.

*Autopsy.*—The pancreas remnant, somewhat nodular and atrophic, weighed 1.65 gm. The liver appeared fatty, presumably from the high proportions of fat in the diet. The autopsy was otherwise negative except for emaciation.

*Microscopic Examination.*—There was moderate fatty infiltration in the liver; no noteworthy changes in the spleen, kidneys, adrenals, testes, thyroid, parathyroids, and hypophysis. The pancreatic parenchyma mostly showed inter-acinar fibrosis, in the form of light bands traversing it irregularly and often distorting acini. The fibrosis was presumably the result of damage from an acute inflammation following operation; there were no signs of recent or progressive changes. Islands were scarce and small to a markedly pathological degree. In some considerable areas free from fibrosis there was this same scarcity of islands, and the sections were made up of almost unbroken expanses of acini. No vacuolation was visible.

The pancreas remnants in these animals were small, and there was apparently further destruction of islands by inflammation. Diabetes was more or less completely suppressed by fasting and undernutrition, but as neither dog became able to tolerate a diet sufficient to support life, they both died in a little over 2 months of inanition. Very slight hydropic changes were demonstrable in the islands in the first dog, which had shown frequent traces of glycosuria, but none were found in the second dog, in which glycosuria had been more rigorously controlled. These examples are comparable with a few human cases of great severity, especially when the treatment consists in continual attempts to push the diet to the limit of tolerance.

#### *Originally Mild Diabetes; Death from the Undernutrition Made Necessary by Prolonged Slight Overfeeding.*

*Dog B2-71.*—Female; mongrel; yellow; age 3 years; good condition; weight 14.7 kilos. June 3, 1914. Removal of pancreatic tissue weighing 19.2 gm. Remnant about main duct estimated at 2.25 gm. ( $\frac{1}{4}$ ). No glycosuria ensued on meat diet. On change to bread and soup, there was the usual single day of glycosuria, thereafter none. The following tolerance tests were performed while the animal was at a fairly constant weight of 13 to 13.5 kilos, no food being given on the test days.

June 26. Subcutaneous injection of 42 gm. of Merck anhydrous glucose in 30 per cent solution (3 gm. per kilo on basis of normal weight of 14 kilos). No glycosuria. July 8. Subcutaneous injection of 56 gm. of Merck glucose in 30 per cent solution (4 gm. per kilo on basis of 14 kilo weight). No glycosuria. July 17. Subcutaneous injection of 84 gm. of Merck glucose in 30 per cent solution (6 gm. per kilo on basis of 14 kilo weight). Glycosuria 0.3 per cent in 10 cc.

of urine; thereafter negative. July 30. 56 gm. of Merck glucose in 30 per cent solution given by stomach tube (4 gm. per kilo on basis of 14 kilo weight). No glycosuria. Aug. 12. 84 gm. of Merck glucose in 30 per cent solution given by stomach tube (6 gm. per kilo on basis of 14 kilo weight). 3 hours after dose, moderate sugar reaction in 1 cc. of urine from bladder; next morning trace in 420 cc.

The subsequent record is contained in Table I.

TABLE I.  
Dog B2-71.

Date.	Body weight.	Diet.	Remarks
<i>1914</i>			
Aug. 21-23	13.8	Bread and soup with 100 gm. of glucose daily.	No glycosuria.
" 24-26	13.9	Bread and soup with 200 gm. of glucose daily.	" "
" 27- Sept. 26	14-11	Bread and soup with 300 gm. of glucose daily.	Glycosuria varying from heavy to negative; generally slight. Diarrhea; decline of weight and strength.
Sept. 27- Oct. 27	11-12	Bread and soup <i>ad libitum</i> .	Occasional traces of glycosuria, governed by variations of appetite, increasing with the slight gain in weight.
Oct. 28- Nov. 7	12 1-11.8	Bread and soup first with 50, then with 100 gm. of glucose daily.	Heavy glycosuria, diminishing as appetite failed.
Nov. 8- Jan. 2, 1915	11.8-13 8	Bread and soup with a little meat and suet daily.	Glycosuria absent at first, then irregular traces, becoming heavy at end with gain in weight.
<i>1915</i>			
Jan. 3-6	13.8-13.1	Fasting.	Glycosuria absent.
" 7-	13.5-12.6	Beef lung <i>ad libitum</i> .	" "
Mar. 28			
Mar. 29- July 2	12.6-10.8	1 kilo of beef lung daily.	No glycosuria except on days of carbohydrate feeding, as recorded in Paper 2,* which showed tolerance between 100 and 200 gm. of bread or rice.
July 3- Aug. 3	10.8-12.5	Beef lung <i>ad libitum</i> with 50 gm. of suet daily.	No glycosuria except in occasional experiments, which showed that tolerance was less than 50 gm. of bread.

\* Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 399.

The dog was then used for experiments unrelated to the present subject. Prior to death on Nov. 24, 1915, glycosuria was beginning to appear on a carbohydrate-free diet of beef lung and suet.

*Autopsy.*—The important feature of the autopsy was the pancreas remnant, which weighed 4.1 gm.

*Microscopic Examination.*—The islands were unusually large and numerous, and contained a slight sprinkling of maximally vacuolated cells, evidently related to the overfeeding preceding death.

The record represents downward progress during approximately a year and a half of life after operation. The animal was never subjected to any heavy continuous glycosuria. Certain fluctuations of tolerance were related to changes of weight, as noted in Table I, but there was no obesity and the weight remained always below the original normal level. The dog was merely kept fed as close as possible to the limit of tolerance, up to January, 1915, on bread, thereafter on meat, and tests or experiments were frequently interpolated producing slight or transitory glycosuria. Slow decline of assimilation thus occurred notwithstanding hypertrophy of the pancreas remnant and its richness in islands.

This finding, together with a number of animal and human observations, suggests a functional injury or defect in the islands when their number and size seem sufficient to prevent diabetes. The degree of vacuolation as usual corresponded to the glycosuria.

The chief point in the experiment is its close imitation of human cases in which the diet is forced to the verge of tolerance and the patient commits occasional indiscretions causing transitory glycosuria. The familiar downward progress of such cases is accurately reproduced.

*Dog B2-31 (Fig. 4).*—Female; bull-terrier; white; age 2 years; good condition; weight 11 kilos. Dec. 23, 1913. Removal of pancreatic tissue weighing 19 gm. Remnant about main duct estimated at 2.6 gm. ( $\frac{1}{2} - \frac{1}{4}$ ). The dog was able to live on bread and soup with only slight intermittent glycosuria. Her gluttony and fondness for sugar made diabetes possible, as described in Paper 2.<sup>1</sup>

Mar. 8, 1914. Fasting was begun. Mar. 12. Glycosuria was absent. Nevertheless, the fast was continued through Mar. 16. Mar. 17. The body weight was 8 kilos, and very low weighed diets were begun, generally 50 gm. of lung and

---

<sup>1</sup> Allen, F. M., *J. Exp. Med.*, 1920, **xxi**, 382.

50 gm. of suet, reducing the weight to 7.4 kilos on Mar. 25. These diets also were given in the form of meals spaced as far apart as possible, in the hope of better utilization from the standpoint of diabetes and also of the protein economy in the sense of Thomas,<sup>2</sup> though no plain evidence of the benefit of such a course was ever seen. Beginning Apr. 2, the daily ration was increased to three meals of 30 gm. of lung and 30 gm. of suet each. By Apr. 24 the weight had risen to 9.3 kilos, and the diet was increased to 40 gm. of lung and 40 gm. of suet thrice daily.

Apr. 27, 4 p.m. A tolerance test was performed by subcutaneous injection of 11 gm. of Merck anhydrous dextrose (1 gm. per kilo on the basis of the normal weight of 11 kilos) in 30 per cent solution. 10 p.m. Urine by catheter was 128 cc. with 0.9 per cent glucose. Apr. 28, a.m. Urine by catheter was 388 cc. with 0.4 per cent glucose; i.e., an excretion of 2.7 gm. and utilization of 8.3 gm. of the dose.

Glycosuria was then absent till May 1, when the body weight was 9.9 kilos. It was continuous for 3 days, May 1 to 3, but not above 0.4 per cent. After fast days on May 3 and 4, the same diet was resumed, with glycosuria absent except as follows: May 7, 0.2 per cent, stopped by a fast day; May 17, 0.3 per cent, stopped likewise; May 24, 0.5 per cent, stopped by 2 fast days. After this the diet was reduced to 20 gm. of lung and 30 gm. of suet thrice daily. Nevertheless, on May 30, at a body weight of 9.75 kilos, there was a return of 0.17 per cent glycosuria. Though this ceased within 24 hours, the fast was continued for 5 days.

Thereafter, on a diet of 30 gm. of lung and 30 gm. of suet thrice daily, there was a gradual gain of weight up to 10.8 kilos on July 3, when there appeared glycosuria of 0.46 per cent in 600 cc. of urine, increasing to 0.97 per cent in 835 cc. of urine on July 4.

Fasting was imposed till July 7, when the weight was 10.3 kilos. Thereafter 100 gm. of lung were fed daily. Though the weight fell slightly on this lower caloric diet, there was continuous slight glycosuria on July 11 to 13, not above 0.66 per cent. The diet was then changed to 100 gm. of fresh beef pancreas daily, and the same glycosuria (not above 0.7 per cent) continued till July 20, again illustrating the uselessness of pancreas feeding.

After a 4 day fast, the weight was down to 9.3 kilos on July 24, and the diet of 100 gm. of raw pancreas was resumed, together with 30 gm. of bone-meal, which was commonly used for such experiments, as previously stated. In addition to the usual purpose of preventing diarrhea and supplying salts to the body, the bone-meal might perhaps serve in this connection to neutralize gastric juice. July 28. There began an excretion of 1.5 per cent glucose in 425 cc. of urine, increasing to 4 per cent in 360 cc. of urine the next day. Fasting then stopped the glycosuria in 2 days, but was continued to Aug. 4, when the weight was 8.5 kilos. The diet of 100 gm. of pancreas was then resumed, with resultant glyco-

<sup>2</sup> Thomas, K., *Arch. Physiol.*, 1910, suppl., 249.

suria of 0.2 to 0.4 per cent on Aug. 6 to 8. Fasting was then instituted for 4 days. As it seemed evident that the rigorous undernutrition was beginning to be effective in diminishing glycosuria, a change was made to lung diet, to guard against ascribing any benefit incorrectly to the pancreas feeding. Accordingly, beginning Aug. 11, 100 gm. of lung were fed daily, with absence of glycosuria except for a trace on 1 day. The weight at this time was 8.15 kilos.

Aug. 18. Bread and soup were given by mistake, with resultant glycosuria of 6.6 per cent in 1,275 cc. of urine. Fasting till Aug. 25 was necessary before glycosuria ceased. Thereafter on a diet of 20 gm. of lung and 20 gm. of suet thrice daily, glycosuria was absent except for a trace on 1 day.

Sept. 11. As the dog had tired of fat, the diet was changed to 50 gm. of lung thrice daily. Sept. 13. Glycosuria of 1 per cent appeared, the higher protein as usual having a greater immediate effect than the higher calories. Sugar excretion was continuous on this diet, and by Sept. 26 had risen to 6 per cent. Accordingly, fasting was begun on Sept. 27; glycosuria was absent on Oct. 4; nothing more than 60 gm. of suet was given daily till Oct. 9, when a diet of 50 gm. of lung and 50 gm. of suet was begun, and increased on Oct. 16 to 80 gm. of lung and 80 gm. of suet. The weight was then 6.7 kilos.

Slight glycosuria, not above 0.5 per cent, occurred on Oct. 22, 23, 28, 29, 30, and Nov. 6. Suet was then omitted, but similar sporadic traces continued till Nov. 23, when 2 fast days were imposed, followed by a diet of 50 gm. of lung and 30 gm. of suet. The weight rose slightly, to 6.9 kilos on Dec. 9, on which day glycosuria of 0.6 per cent appeared.

With 2 days of fasting, the urine was sugar-free on Dec. 11. Dec. 12. A diet of 50 gm. of lung was begun, with the addition of 30 gm. of suet after Dec. 18. About this time the previously lively animal began to fail seriously. The digestion began to be impaired, as usual in cachexia, and by Dec. 31 the weight was down to 5.8 kilos. The diet was changed to 50 gm. of raw pancreas and 30 gm. of suet, in the hope of improving digestion, but the pancreas was of no avail even for this purpose. Liberal feeding with pancreas and fat was attempted on Jan. 2 and 3, 1915; the result was a glycosuria of 1 per cent on Jan. 4, while the weight had fallen to 5.3 kilos. The dog was chloroformed.

*Autopsy.*—The urine in the bladder was 13 cc., containing 0.53 per cent glucose, a trace of albumin, and no acetone. Except for emaciation and corresponding atrophy of viscera, the autopsy was negative as usual. The weights of the principal organs were as follows: liver 101 gm.; both kidneys 48.8 gm.; both adrenals 2 gm.; pancreas remnant 3.8 gm. The pancreas remnant was soft, lobulated, and in places semitranslucent.

*Microscopic Examination.*—The liver showed a considerable sprinkling of glycogen granules by Best's carmine stain; these were located in the periphery of the lobules, the centers containing none; fat was scanty. The acinar tissue of the pancreas was normal. Islands were remarkable for both fewness and smallness. Their cells were normal in appearance and no vacuolation was discoverable.

Notwithstanding the extreme emaciation and prostration, glycosuria evidently continued till death. The point is mentioned because dogs of this type herein correspond to the rule for human cases. In some totally depancreatized dogs authors have reported cessation of glycosuria before death.

In spite of the severity of diabetes thus indicated, no sign of hydropic degeneration was visible in the islands of Langerhans. Study of a sufficient series of animals gives a simple explanation. The overstimulation causing such visible degeneration is intense and the cellular disintegration rather rapid. The diabetes in this dog had been largely controlled by diet. Life had continued for more than a year, and an active hydropic process must have destroyed all the islands before this time. The milder degree of overstimulation and longer clinical course should naturally give rise to a slower island destruction, in the form of occasional loss of cells, which would seldom be demonstrable microscopically. This supposition, which in animals can be fully verified by observations of all types, degrees, and stages of the process, serves to explain the similar findings in human cases.

The principal reason for the detailed record given is to show the exact similarity to the clinical course of many human cases. The animal started with mild diabetes; in fact, the large size of the pancreas remnant and the subsequent hypertrophy would probably have permitted a cure had opportunity been given. Gluttony and carbohydrate excess made the diabetes severe. A familiar plan of treatment was then employed, in the form of a protein-fat diet pushed to the point of maintaining the highest possible weight and strength. There was the usual onset of glycosuria with gain in weight, the necessity for repeated fasting periods, and the gradual decline of tolerance, weight, and strength. This occurred without any breaking of diet on the part of the subject. The two principal features of cases under such management are thus illustrated; first, life and strength are preserved much longer than when active diabetic symptoms are allowed to continue; second, the alleged "spontaneous" downward progress of human patients occurs in typical slow but sure form.

*Glycosuria Uncontrollable after Protein Overfeeding.*

*Dog B2-56.*—The record of this animal was given in Paper 3<sup>8</sup> as an example of downward progress on excessive protein diet. The attempt to control the condition by fasting beginning May 14, 1914, gave results shown in Table II.

The cachexia was unusually rapid in progress, and even before the fast the dog had lost considerable hair and was developing ulcers at points of bony pressure, especially over the ischiatic tuberosities and

TABLE II.

*Dog B2-56.*

Date.	Urine.		Remarks.
	Volume. cc.	Glucose. per cent	
1914			
May 14	840	1.7	Weight 13.5 kilos. Fasting.
" 15	490	3.0	Fasting.
" 16	530	3.5	"
" 17	415	1.6	"
" 18	450	1.0	"
" 19	360	1.5	"
" 20	267	1.4	"
" 21	397	1.5	"
" 23	430	1.3	"
" 24	535	2.4	Weight 9.9 kilos. Diet of meat <i>ad libitum</i> begun because of dangerous weakness.
" 25	1,000	0.7	Diet of meat <i>ad libitum</i> .
" 26	1,150	0.8	" " " "
" 27	690	0.6	" " " "
" 28			Weight 9.7 kilos. Moribund. Killed for autopsy.

the joints of the limbs. These were foul and spreading in character, without tendency to heal. The one at the right elbow perforated into the joint and was probably the origin of the large axillary abscess of creamy pus found at autopsy. It is unknown why a few partially depancreatized dogs show cachexia and susceptibility to infection almost like totally depancreatized animals. The "gangrene" and lowered resistance of human patients are fully reproduced in such animals. Also in occasional human cases the diabetes is evidently too severe to be controlled by fasting.

<sup>8</sup> Allen, F. M., *J. Exp. Med.*, 1920, **xxxi**, 560.

*Glycosuria from Prolonged Protein-Fat Overfeeding, Checked at First by Short Fasting, Later Uncontrollable.*

*Dog B2-57.*—Female; mongrel; yellow; age 5 years; good condition. May 4, 1914. Received at normal weight of 11 kilos. After reduction of weight to 8.65 kilos by fasting, on May 21 partial pancreatectomy was performed; 19.6 gm. of tissue removed, and remnant left estimated at 3 gm. The dog was used to test the effects of fat feeding and obesity. The first continuous glycosuria began Dec. 24, at a weight of 16.3 kilos. This persisted till stopped by fasting, Jan. 4 to 6. The record for 1915 is given in Table III.

*Dog B2-30.*—Female; mongrel; yellow; age 1½ years; good condition; weight 10 kilos. Dec. 22, 1913. Removal of pancreatic tissue weighing 19.5 gm. Remnant about main duct estimated at 2.5 gm. Glycosuria was absent after operation till milk was fed on Dec. 26. It then remained heavy (up to 3.3 per cent) on bread and soup diet till Jan. 1, 1914, after which a large (unweighed) admixture of glucose was required to produce glycosuria. The dog gradually reached the point of refusing the mixture absolutely; therefore on Jan. 17 the diet was changed to chopped meat mixed with lactose, which was eaten abundantly and maintained glycosuria as high as 5 per cent. After Jan. 22 heavy glycosuria continued on a diet of meat only. Feb. 2. Fasting was begun in the attempt to stop glycosuria (Table IV).

*Blood Transfusion.*—Feb. 20. Transfusion was undertaken partly in an attempt to increase strength and partly to determine whether, when everything possible was done by fasting, there might be any transfer of pancreatic hormone in the blood sufficient to check the diabetes. 4.30 p.m. Urine under the cage was 25 cc., containing glucose 4.8 per cent. When all was ready for transfusion, catheterization yielded 2 cc. of urine, with glucose 6.2 per cent. A large needle was then inserted into an external jugular vein, and by the use of vaseline-coated syringes without anticoagulants blood was drawn from a normal dog and injected into this dog to the amount of 350 cc. by 5.30 p.m. At this time the dog showed collapse, passed urine and feces, and seemed on the point of death, from toxic action of the infused blood or simple overfilling of the circulation. The transfusion was therefore ended, and the emptiness of the bladder verified with the catheter. The total urine for the transfusion period was 3 cc., containing 6.4 per cent glucose. 6.15 p.m. The urine obtained by catheter was 4 cc., containing 3 per cent glucose and slight albumin. The dog drank thirstily. 8.30 p.m. The urine by catheter was 19 cc., with a faint trace of albumin and 1.7 per cent sugar. 2.30 a.m. The urine by catheter was 26 cc. with 2.5 per cent glucose and the same faint albumin. The mere traces of glycosuria on the succeeding days, as shown in Table IV, may appear striking, but the writer is inclined to interpret them as accidents of renal permeability rather than benefit to the diabetes.

TABLE III.  
Dog B2-57.

Date.	Body weight.	Diet.	Urine.	
			Volume.	Glucose.
			cc.	per cent
1915				
Jan. 1	16.3	100 gm. of lard and 1,000 gm. of beef lung, the latter not all eaten.	650	1.5
" 2		100 gm. of lard and 1,000 gm. of beef lung, the latter not all eaten	880	4.2
" 3		Most of food uneaten.	890	1.6
" 4		Fasting.	180	0.7
" 5	15.4	"	339	0.22
" 6		"	261	0
" 7	14.8	"	113	0
" 8	14.5	"	56	0
" 9	14.3	100 gm. of lung.	196	0
" 10		200 " " "	194	0
" 11	14.0	300 " " "	231	Doubtful.
" 12	14.13	400 " " "	280	0
" 13	13.73	500 " " "	203	0
" 14	13.55	500 " " "	186	0.18
" 15	13.60	600 " " "	239	1.37
" 16	13.75	700 " " "	107	1.33
" 17		Fasting.	81	0
" 18	13.30	"	65	0
" 19	13.20	500 gm. of lung.	119	0.7
" 20	13.05	500 " " "	369	0.23
" 21	12.86	Fasting.	165	Doubtful.
" 22	12.60	"	154	0
" 23	12.45	400 gm. of lung.	250	0.20
" 24		400 " " " 30 gm. of suet.	386	2.50
" 25	12.40	Fasting.	14	0.90
" 26	12.18	"	99	0.60
" 27	12.06	"	95	Very faint.
" 28	11.90	"	50	0
" 29	11.75	100 gm. of lung.	170	0
" 30	11.75	150 " " "	350	2.80
" 31		Fasting.	273	2.00
Feb. 1	11.30	"	142	1.50
" 2	11.15	"	211	0.80
" 3	11.10	"	195	1.00
" 4	10.85	"	185	0.70
" 5	10.68	"	81	0.60
" 6	10.61	"	89	2.00
" 7		"	183	1.25

TABLE III.—*Concluded.*

Date.	Body weight.	Diet.	Urine.	
			Volume.	Glucose. per cent
1915	kg.		cc.	
Feb. 9	10.25	Fasting.	176	3.00
"	10.10	"	204	2.78
" 10	9.90	"	185	2.00
" 11	9.80	"	70	2.00
" 12	9.50	"	116	1.20
" 13	9.50	50 gm. of glidine.	295	3.03
" 14		Fasting.	135	1.40
" 15	9.30	"	102	1.70
" 16	9.15	"	105	2.50
" 17	9.00	"	175	2.50
" 18	8.77	40 gm. of extract of beef.	385	2.53
" 19	8.78	Fasting.	274	1.33
" 20	8.85	50 gm. of lard; 50 gm. of meat.	260	1.92
" 21		50 " " " 50 " " "	120	1.41
" 22		50 " " " 50 " " "	219	0.72
" 23	8.25	50 " " " 50 " " "	570	0.30
" 24	8.10	50 " " " 50 " " "	800	0.69
" 25	8.10	50 " " " 50 " " "	752	0.72
" 26	7.85	50 " " " 50 " " "	747	0.80
" 27	7.80	50 " " " 100 " " "	1,295	0.90

*Subsequent History of Dog B2-30.*—After Mar. 21, the dog was given 25 gm. of lung and 25 gm. of suet two or three times daily; *i.e.*, a diet of either 50 or 75 gm. each of lung and suet. Apr. 2. At a body weight of 5.5 kilos, glycosuria of 2.7 per cent appeared, and was stopped only by fasting till Apr. 5. The diets thereafter were lower than before, sometimes only 10 gm. of lung and 10 gm. of suet thrice daily. With advancing cachexia the digestion became poor; glycosuria was absent, but by Apr. 21 the weight had fallen to 4.3 kilos, and the animal could barely stand. Accordingly, on that date it seemed necessary to give four meals of 40 gm. of lung each, without suet. The result was an immediate glycosuria of 1.7 per cent in 140 cc. of urine. Though the diet was increased to 50 gm. of lung four times daily, which was as much as the dog would take, glycosuria ceased through failure of digestion. Apr. 24. The animal was at the point of death and was chloroformed.

*Autopsy.*—Performed immediately. Aside from the extreme emaciation and accompanying atrophy of viscera, the autopsy was negative. The pancreas remnant, normal in appearance and consistency, weighed 3.9 gm.

*Microscopic Examination.*—The acini were normal, uneven in the degree of filling, but mostly containing abundant zymogen. Island tissue was scarce

TABLE IV.  
Dog B2-30.

Date.	Body weight.	Urine.		Dextrose-nitrogen ratio.
		Glucose.	Nitrogen.	
1914				
Feb. 2	8.6	9.25		
" 3				
" 4	8.1			
" 5	7.8	1.25		
" 6	7.8	2.21		
" 7	7.8	3.42	3.14	1.09
" 8		0.92	3.11	0.29
" 9	7.5	1.48	2.81	
" 10	7.25	0.60	2.69	0.22
" 11	7.15	4.45	3.25	1.37
" 12		2.22	2.67	
" 13	6.90	0.06	2.60	
" 14	6.80	Faint.	1.93	
" 15		"	2.48	
" 16	6.55	0.64	3.40	
" 17	6.50	1.02	2.08	0.49
" 18	6.30	0.62	1.80	0.34
" 19	6.20	2.83	1.73	1.63
" 20	6.08	3.68	2.44	1.51
" 21	6.40	Faint.	2.43	
" 22	6.23	"	2.63	
" 23	6.02	4.13	3.82	1.08
" 24		7.32	4.99	1.47
" 25	5.65	2.82	3.53	0.80
" 26	5.60	Faint.	3.41	
" 27	5.43	0	4.66	
" 28	5.45	0	3.30	
Mar. 1	5.35	0	4.44	
" 2	5.20	0	3.01	
" 3	5.18	0	3.07	
" 4	5.20	0	2.42	
" 5	5.38	0	2.74	
" 6	5.28	0.90	3.14	
" 7	5.33	0.24	2.20	
" 8	5.28	Very faint.	2.28	
" 9	5.20	0	1.31	
" 10	5.15	0	1.98	
" 11	5.23	0	2.06	
" 12	5.23	0	1.54	
" 13	5.20	0	1.87	
" 14	5.20	0	1.97	
" 15	5.28	0	1.89	
" 16	5.30	0	1.97	
" 17	5.25	0	1.89	
" 18	5.28	0	2.11	
" 19	5.20	0	2.75	
" 20	5.20	0	2.20	

almost to the vanishing point. Search revealed occasional tiny clumps of cells of island character. No Bensley stains were made, but by comparison with other animals in which such stains were made these surviving, non-vacuolated cells may be interpreted as alpha cells, in confirmation of the original observations by Homans.<sup>4</sup> In rare instances such cell clumps contained one to three maximally vacuolated cells, presumably the last remains of degenerated beta cells. As usual, the islands were not replaced by fibrosis; the tissue merely gave the impression of unbroken expanses of acini.

Starting with sugar-free urine on January 1 on bread and soup diet, this dog showed a downward progress which was so rapid that within 1 month the pancreatic function had fallen too low to support life.

The dextrose-nitrogen ratio was never maximal and was generally low. It is known from the literature that even totally depancreatized dogs do not necessarily show the full 2.8 ratio during fasting. Partially depancreatized dogs of the present type seldom or never show this full ratio during fasting, yet the absence of it is no proof that life can be maintained.

The occasional temporary cessation of glycosuria in this animal during fasting is not unusual under these circumstances and is presumably due to changes of renal permeability. It is probable that the blood sugar remained high till about the time of death.

Transfusion of blood improved the strength slightly and perhaps altered renal permeability temporarily, but had no positive effect upon the diabetes. In view of the similar result in a child,<sup>5</sup> it may be concluded that the effect of transfusion is negative.

The evidence of the hopelessly low pancreatic function was that with the lowest possible diets and body weights, lasting freedom from glycosuria could not be achieved, so that the animal necessarily died from weakness.

The extreme degree of exhaustion and disappearance of island tissue correspond to the lowered function as mentioned.

<sup>4</sup> Homans, J., *J. Med. Research*, 1914, xxx, 49; 1915, xxxiii, 1.

<sup>5</sup> Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, Chapter III, Case 45, 361.

*Dog B2-01 (Figs. 2 and 3).*—Part of the history of this animal was given in Paper 3. The terminal period so closely resembles that of Dog B2-30 that it can be summarized here very briefly, particularly because it supplements the

TABLE V.  
*Dog B2-01.*

Date.	Urine.						Plasma sugar. per cent	Remarks.
	Volume. cc.	Glucose.		Total ni- trogen. gm.	Dextro- nitrogen ratio.	Nitropruss- ide reaction.		
1919								
Oct. 22	145	2.20	3.19	4.66	0.68	Heavy.	0.432	Weight 13.3 kilos. Fasting.
" 23		Heavy.				"		"
" 24	155	1.80	2.79			"		"
" 25		Heavy.				"		"
" 26	65	2.29	1.48			"		"
" 27		Heavy.				"		"
" 28	118	1.15	1.36			"		"
" 29	50	0.95	0.48	0.98	0.49	Moderate.		"
" 30	110	0.45	0.50			"		"
" 31	200	Moderate.				"		"
Nov. 1	200	0.18	0.36	1.72	0.21	Slight.		"
" 2		Slight.				"		"
" 3		Faint.				"		"
" 4		0				"		"
" 5		0				Negative.	0.322	Fed 50 gm. of tallow.
" 6		0				"		" 100 " " "
" 7		0				"		Refuses fat. Fed bones.
" 8	1,500	0.80	12.0	5.6	2.1	Negative.		Fed 100 gm. of beef.
" 9	270	0.55	1.5	3.2	0.47	"		Fasting.
" 10	190	0.25	0.48			Slight.		"
" 11	275	Slight.		1.40		Moderate.		"
" 12	200	"		1.30		Slight.		"
" 13		"				Negative.		"
" 14	210	0.40	0.84	2.03	0.41	"		"
" 15		Slight.				"		"

other by its blood sugar analyses, which were lacking in Dog B2-30. Heavy sugar and acetone reactions being found present on the diet of lung and suet as stated,<sup>6</sup> and the blood plasma on Oct. 16, 1919 showing 0.520 per cent sugar and a slight nitroprusside reaction, fasting was begun on this date in the

<sup>6</sup> Allen,<sup>8</sup> p. 570.

attempt to control the diabetes, though with little expectation of success. After Oct. 22, the seriousness of the condition being confirmed by the stubborn persistence of glycosuria, occasional quantitative analyses of sugar and nitrogen were performed (Table V). Urine was voided without catheterization, and the analyses apply to such daily specimens, not to accurate 24 hour specimens.

Nov. 15, p. m. The dog was dying. An intraperitoneal saline injection given after taking blood at 8 p.m. failed to benefit. 10 p.m. Animal found dead. The results of blood analyses are shown in Table VI.

This animal was one of those in which the severity of diabetes is too great to be checked by fasting. This condition is more common in experimental than in clinical diabetes; but is by no means unknown in the latter. The greater susceptibility of human patients to acidosis

TABLE VI.

Dog B2-01.

Date.	Plasma sugar. per cent	Blood urea per 100 cc.	Plasma chlorides per 100 cc.	Plasma CO <sub>2</sub> capacity. vol. per cent	Plasma nitroprusside reaction.
1919					
Oct. 16	0.520	23.4	550	59.8	Moderate.
" 22	0.432				Slight.
Nov. 5	0.322	26.0	533	56.6	Negative.
" 14	0.624	71.4	562	62.4	Slight.
" 15, 10 a.m.	0.872				Negative.
8 p.m.	0.600	38.6	536	59.4	"

is the probable reason why, with hopelessly severe diabetes, they generally die quickly in coma rather than in the prolonged cachexia which is characteristic of dogs. It will be shown later that under suitable conditions dogs also reach a state where they develop coma with either feeding or fasting.

It is not known whether the D:N ratio in this animal was ever maximal. The high ratio of 2.1 on November 8, in consequence of only a single feeding of 100 gm. of beef, suggests that the full 2.8 ratio might have been present on feeding; but on the other hand, the unusual glycosuria on this date may have represented partly a mere sweeping out of retained sugar. The most striking feature is the remarkably low sugar excretion and D:N ratios in a hopelessly severe case of diabetes. Such submaximal ratios have been the rule in other dogs of this sort during fasting.

Renal impermeability, which is a familiar phenomenon in many human patients, seemed to be largely responsible for the low and variable glycosuria. Absence of glycosuria, on November 4 to 7, with plasma sugar as high as 0.322 per cent, was similar to what has been observed in several other animals under similar conditions, and could not be interpreted as indicating that the diabetes was under control. It may be noted that this impermeability existed in an animal without histological signs of nephritis at autopsy, and with no known cause of impaired renal function other than the diabetes.

The apparent renal impermeability did not include retention of chlorides so far as indicated by the blood analyses. The blood urea rose to a strikingly high figure on November 14, but fell before death in a manner difficult to explain by simple retention. The observations concerning urea also closely reproduce the conditions found in certain human patients.

Nitroprusside reactions were heavy in the urine and slight in the plasma on beginning fasting, and diminished during the fast, as usual in human patients. This clearing of the acetone bodies was approximately parallel with the decline of glycosuria. These tests became negative during the period of freedom from glycosuria, and returned after glycosuria had been restored by protein feeding. They were negative preceding death, as often happens in cachexia. The principal point of these observations is that in this hopelessly diabetic animal the sugar, whether retained from renal impermeability or any other cause, seemed somehow to be used in a way to prevent acetoacetic acid formation.

*Dog C3-56 (Fig. 9).*—Female; mongrel; black and white; age 3 years; moderately well nourished; weight 15.3 kilos. Jan. 27, 1916. Received. Raised a litter of pups in laboratory. July 13. Removal of pancreatic tissue weighing 35 gm. Remnant about main duct estimated at 4.6 gm. (about  $\frac{1}{3}$ ). Glycosuria occurred on bread and soup feeding, but later required glucose for its continuance. The tolerance was thus broken down so that by the middle of Aug. glycosuria was present on a diet of 1 kilo of beef lung. High fat diets were then given, for purposes and with results explained in detail in a previous publication.<sup>7</sup> The table there shown carried the record up to Nov. 4. The condition then was one of persistent and stubborn hyperglycemia without glycosuria,

<sup>7</sup> Allen, F. M., *Am. J. Med. Sc.*, 1917, cliii, 349, 364.

such as excessive fat diets often produce in human patients. The utmost effort was then made to save the animal by diets such as those used for human cases. The diet of 200 gm. of lung daily was continued to Nov. 18, with the purpose of sparing body protein and conserving strength as well as possible, while building up tolerance by undernutrition. On Nov. 18 the plasma sugar was still 0.208 per cent, though the weight had fallen to 7.6 kilos, and the animal was weak. It was therefore deemed necessary to reduce the protein, and the diet was changed to 75 gm. of lung and 75 gm. of suet, with the addition of one raw egg daily to contribute variety and supply any possible need for fat-soluble vitamine. As the appetite failed, other forms of cooked and raw meat were substituted for variety in the same quantity. By Dec. 3 all food was refused. The urine remained free from sugar and acetone as it had been throughout this entire period, but the plasma sugar of the moribund animal was still 0.137 per cent and the carbon dioxide capacity 37.6 volumes per cent, with analyses for all three acetone bodies negative and all other signs of acidosis absent. The dog then weighed 7.25 kilos in a condition of extreme cachexia, with spreading and perforating ulcers on the legs suggestive of diabetic "gangrene." The animal was therefore killed for autopsy.

*Autopsy.*—The emaciated cadaver contained no visible fat. The liver was small, not fatty, weighing 218 gm. The kidneys, pale and edematous appearing, weighed together 56.5 gm. The viscera were otherwise negative except for atrophy. The pancreas remnant, normal in appearance and consistency, weighed 4.25 gm.

*Microscopic Examination.*—The organs were negative except for the usual vacuolation of renal tubules due to glycogen or fat. The pancreas remnant, free from fibrosis, consisted of almost unbroken expanses of small acini, some entirely empty but the majority containing zymogen. Only the last remains of exhausted islands were found, in the form of small clumps of cells, sometimes maximally vacuolated (presumably beta cells), sometimes non-vacuolated (presumably alpha cells), sometimes a mixture of the two. There was also vacuolation in the cells of some of the small ducts, as sometimes found in this extreme terminal stage of diabetes.

The experiment illustrates hyperglycemia and impairment of assimilation brought on chiefly by fat feeding, and downward progress notwithstanding absence of glycosuria. Conditions in many human cases are thus closely imitated.

The hydropic degeneration of islands with hyperglycemia without glycosuria is also illustrated. Under these as under all other circumstances, the tendency is for canine diabetes to run a more rapid course than human diabetes. For this reason hydropic degeneration is always easier to demonstrate in dogs, but may be assumed to occur

more slowly in human patients who show a similar but slower downward progress with hyperglycemia.

The clinical and anatomic findings combine to support the view that the internal pancreatic function was here too deficient to support life. A similar conclusion may be drawn from similar evidence in some of the worst human cases.

One point worth mentioning is that diabetic dogs are not necessarily wretched and cachectic. Especially those possessing a maximum of pancreatic tissue, and best suited for therapeutic and some other tests, may be normal appearing and also comfortable and happy. The other point of importance is the degree to which the health and life of these animals is dependent on their diet. These facts are shown more plainly by photographs (Figs. 1 to 12) than by any verbal description, and also the parallelism with the conditions of human patients is more clearly illustrated.

#### CONCLUSIONS.

1. Practically every detail of clinical diabetes can be reproduced in partially depancreatized animals. The resemblance is made still more exact by the susceptibility of such dogs to acidosis and coma, as will be shown in later papers, and also by the similarity of the anatomic changes in the islands of Langerhans. These animals are therefore useful test objects for a therapeutic investigation.

2. These animals at first show considerable tendency to regain assimilation, comparable to that in the early stages of most human cases of diabetes; and in some instances they recover so as to be able to endure any degree or duration of carbohydrate feeding and can be made diabetic only by removal of additional pancreatic tissue. Similar recovery in some human cases, especially after acute pancreatitis, is a probability. This recuperative tendency can be negatived by overfeeding, even without glycosuria. With duration of the diabetes the power of recuperation diminishes and practically disappears in dogs as in human patients.

3. In the absence of progressive pancreatitis or other extraneous causes, these dogs show no inherent downward tendency in their assimilation. This conclusion rests upon observations as long as 6

years from the first pancreas operation and 3 years of known diabetes. This absence of inherent progressiveness is what should naturally be expected in animals with simple resection of part of an organ, and serves further to fit them for accurate feeding experiments.

4. Every detail of the downward progress of human patients on various diets is reproduced in such animals. They lose assimilation and die most rapidly on diets rich in carbohydrate, and less rapidly on excess of other foods. The differences between sugar and starch, and between starch and protein, seem to be only those of degree and time rather than anything absolute. The important point is that, granting the absence of spontaneous downward tendencies as stated, all the different kinds and degrees of downward progress in the records of animals in this and the preceding paper are purely the results of overstrain of the internal pancreatic function by excess of food.

5. Varying degrees of success and failure in the dietetic control of diabetes are also illustrated. The benefit of the classical treatment by exclusion of preformed carbohydrate and limitation of protein is confirmed, in the prolongation of life and well-being to some extent in nearly all cases and perhaps indefinitely in some of the mildest cases. In the great majority of cases such a therapeutic result is not permanent, and downward progress is finally observed if the observations are continued long enough. In most of these cases life, strength, and assimilation can be preserved for a much longer time by a degree of undernutrition suited to the severity of the diabetes, and accomplished by limitation of fat in the diet. The permanence of such control is supported by the unimpaired or rising assimilation in experiments of 1 to  $1\frac{3}{4}$  years duration, but still longer observations would be desirable. Diabetes of great severity is controllable only by correspondingly radical undernutrition. In still more severe cases glycosuria can be abolished only by a degree of undernutrition which entails final death from inanition. In the most severe cases glycosuria cannot be stopped, evidently because the assimilative power is too low to dispose of even the minimum supply of food materials; namely, that derived from the body stores in fasting.

6. A claim of saving every patient, no matter how near death, would be a preposterous one for any remedy in any disease, and the

animal experiments do not support such a claim for diabetes. Also it is unreasonable to expect the actual cure of an organic deficiency by diet, and the diet treatment in animals just as in patients generally represents the sparing rather than the restoration of the weakened function. The basis of the belief in the inherently progressive tendency of severe cases of clinical diabetes is shaken by the exact reproduction of such case histories by diet in animals which are free from spontaneous downward tendencies; but there is still lack of a sufficient number of patients treated on the principle of relief of the total metabolic burden to demonstrate the absence of such inherent progressiveness in human diabetes. As described elsewhere,<sup>8</sup> the principle mentioned has given encouraging results in proportion as it has received actual application in practice. The clinical problem requires the same prolonged careful control of all discoverable symptoms as in animals, and cases too severe for such control, or complicated with infections, violations of diet, etc., are on the same plane as animals in which a similar condition has been produced. Some proportion of cases, especially in young persons, will be found suitable for accurate determination of the question of whether all or most cases of severe diabetes are inevitably progressive and hopeless, and the writer looks forward to publishing such a series. Irrespective of the outcome, the raising of this question is justified by its importance. On the theoretical side, it involves both the general prognosis and the nature of the process underlying diabetes. On the practical side, it is already established that diet is at least the chief cause of downward progress, and it is important to eliminate this cause by avoiding the dietary injuries illustrated in the animal experiments.

<sup>8</sup> Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919.

## EXPLANATION OF PLATES.

## PLATE 65.

FIG. 1. Dog B2-00. The picture was taken while the animal was diabetic in 1917, but shows approximately the condition existing during the 6 years of laboratory life and still maintained on the restricted protein-fat diet.

FIG. 2. The usual sturdy appearance of Dog B2-01 at her ordinary weight between 13 and 14 kilos.

FIG. 3. Dog B2-01 in July, 1917, when fattened with suet to 16.5 kilos and with hyperglycemia present.

FIG. 4. Dog B2-31 in the state of moderate undernutrition which is indicated by the distinctness of individual vertebræ along the back, and which was effective in abolishing glycosuria for several months. The dog had first become diabetic with  $\frac{1}{2} - \frac{1}{3}$  of the pancreas through her gluttony for bread and sugar. Afterward, though undernutrition was maintained, it was not thorough enough. Diets of protein and fat were pushed too close to the verge of tolerance, raising the weight not to normal, but nevertheless higher than the assimilative power could carry. The final result was hopeless diabetes.

FIG. 5. Dog B2-25 in the state of emaciation which had to be maintained for a number of months to control diabetes with only  $1\frac{1}{2}$  to  $1\frac{1}{3}$  of the pancreas present. At this weight of 11 kilos the animal remained strong and lively, and required restraint to keep him quiet for photographing. Through hypertrophy of the pancreas remnant it became possible gradually to raise the weight to 17 kilos, or only 1 kilo below normal.

## PLATE 66.

FIG. 6. Dog B2-79; control to Dog B2-56. The pancreas remnant was  $\frac{1}{8}$ , and microscopically showed more inflammation than that of Dog B2-56. The normal weight of 15 kilos was reduced to 11.7, in which condition 200 to 300 gm. of bread were requisite for glycosuria. The animal is shown 8 months after operation, when a diet of 1 kilo of lung had raised the weight to 15.5 kilos, and glycosuria was accordingly present. The dog was kept in good condition and subjected to several other fluctuations of weight and tolerance, till death 17 months after operation.

FIG. 7. Dog B2-80 at a time when glycosuria had been brought on by fattening to 18.2 kilos on a diet of beef lung and suet. The animal was specially valuable because of excellent digestion due to the large remnant ( $\frac{1}{4} - \frac{1}{3}$  of the pancreas) with which diabetes occurred. The tolerance was high at weights as low as 13.2 kilos. She was used for various observations during 9 months, and the persistent high fat feeding finally brought on not only glycosuria but fatal acidosis. The dog was as sleek and healthy looking as this when she went into typical coma at a weight of 17.25 kilos. Animals of this sort give the closest reproduction of clinical acidosis.

FIG. 8. Dog C3-27 1 week before death in coma, with intense glycosuria, acidosis, and lipemia present; the dog wags her tail, but she was already nauseated and depressed, and the dry nose is a further sign of ill health. The obesity maintained by the high fat diet is evident.

FIG. 9. Dog C3-56 in the closing period. After the damage of high fat diet, the animal was reduced by undernutrition to a feeble state; but though glycosuria was abolished, hyperglycemia was persistent up to death from cachexia. The similar weakness and deficiency of assimilation of certain human patients under similar conditions are well known.

FIG. 10. Dog C3-86 in moderate undernutrition. The fluctuations of tolerance in parallel with the weight were previously described.<sup>9</sup> The dog was vigorous and lively through 1½ years from the time of operation to death.

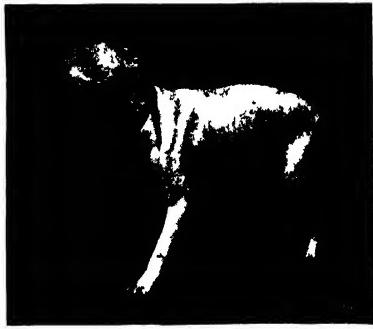
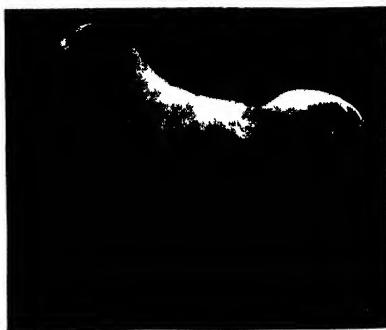
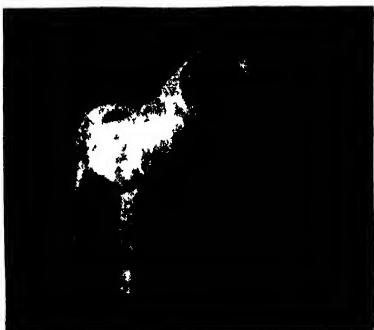
FIG. 11. Dog D4-28, 1½ years after an operation which had left ⅓ of the pancreas. The normal weight was 12 kilos. After prolonged undernutrition, the weight here had been gradually built up to 10.6 kilos. The dog was observed for 1½ years after operation, and remained lively and symptom-free until death from urinary calculus. One feature of the experiment was 50 gm. of bread in the regular ration, showing that a dog with the marked degree of potential diabetes represented by such a small pancreas remnant could by undernutrition be enabled to tolerate such a quantity of carbohydrate for such a length of time without sign of injury.

FIG. 12. Dog D4-52 in Jan., 1918, ½ year after the operation which created mild diabetes. The susceptibility to glycosuria and lowering of assimilation on carbohydrate diet were first demonstrated, and then observations were begun to test whether, with this mild degree of diabetes, a liberal carbohydrate-free diet (with restriction of protein to 500 gm. of beef lung) could be tolerated indefinitely.<sup>10</sup>

---

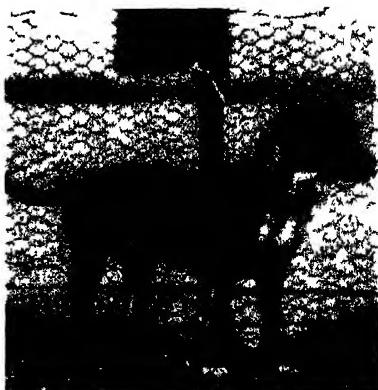
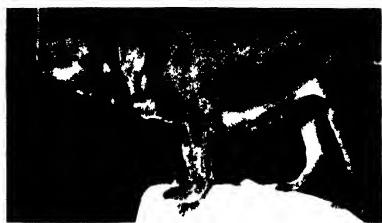
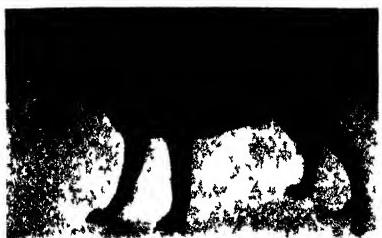
<sup>9</sup> Allen,<sup>7</sup> Chart 8.

<sup>10</sup> Allen,<sup>8</sup> pp. 564-573.



(Allen: Experimental diabetes. I)





(Allen: Experimental diabetes. I)



[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, January 1, 1920, Vol. xxxi,  
No. 1, pp. 35-47.]

## THE CULTURAL DIFFERENTIATION OF BETA HEMOLYTIC STREPTOCOCCI OF HUMAN AND BOVINE ORIGIN.

By J. HOWARD BROWN, PH.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, October 15, 1919.)

Hemolytic streptococci are common in good dairy products and are usually harmless to the consumer. It is desirable to be able to distinguish such streptococci from hemolytic streptococci pathogenic to man which are at times found in dairy products. Up to the present there has been described no qualitative method for distinguishing hemolytic streptococci of human and bovine origin. Our ability to differentiate them depends upon the recognition of certain quantitative differences such as the agglutination or precipitation titer against an immune serum, the rapidity with which hemolysis appears, the size or definiteness of the hemolyzed zone in a blood agar plate, the rate of coagulation of milk, the degree of acidity produced in carbohydrate media, and the action of a bouillon culture on blood corpuscles in suspension. Anything which tends to make the determination of these quantitative differences more evident and simple is of value. Acquaintance with the minute details of appearances in blood agar is useful (1). Avery and Cullen (2) have shown the advantage of the determination of hydrogen ion concentration in dextrose bouillon cultures. We wish to emphasize the differential value of the action of streptococci of human and bovine origin on blood corpuscles in fluid media. The literature on the subject has been reviewed in a previous article.<sup>1</sup>

### *Action of Hemolytic Streptococci on Blood in Fluid Media.*

The Medical Department of the United States Army in 1918 recommended the following method for the identification of *Streptococcus haemolyticus* (3).

<sup>1</sup> Brown (1), Table IV.

"3. 0.5 cc. of the bouillon culture should be mixed with 0.5 cc. of a 5% suspension of washed rabbit blood corpuscles in physiological salt solution and incubated in a water bath at 37°C. for 2 hours. Markedly hemolytic pathogenic streptococci of human origin produce laking of blood under these conditions."

In Table I is recorded the result of the application of the above test to twenty-eight strains of streptococci of bovine and human origin. The strains tabulated were selected as representative of a large number of hemolytic bovine and human streptococci encountered by the author.

Since 1915 I have employed a slightly different technique from that described above for the same purpose and with practically the same results. A fresh young (18 to 20 hours old) standard veal or beef infusion bouillon culture is diluted to twenty times its volume with sterile 0.85 per cent salt solution. To 1 cc. of the diluted culture in small tubes in a Wassermann bath is added one drop of sterile defibrinated blood. The Wassermann tubes should be clean but need not be sterile. 2 hours incubation does not give opportunity for the growth of contaminating organisms to interfere with the reaction. The result of the application of this technique to the same streptococci as employed above is also shown in Table I.

The technique is perhaps a little simpler than that employed by the Army. It must not be assumed, however, that all mixtures of blood or blood corpuscles and bouillon culture will give similar results. It is a purely quantitative test and if it is to be of the maximum differential value the proportions described above must be adhered to. If the culture is diluted 1:10 some of the bovine strains produce hemolysis within the 2 hour period. If it is diluted 1:40 some of the human strains fail to produce hemolysis. Bouillon cannot be used instead of salt solution for making the dilutions. If more than one drop of blood is added the results are irregular. The bouillon cultures must be young and active. Experiments with centrifuged organisms indicate that the number of organisms present is of relatively little importance. The desired result attained by dilution of the culture appears to be due to dilution of the medium rather than dilution of the bacterial suspension. It is necessary to use a greater dilution of the culture when defibrinated blood is employed rather than washed corpuscles because the serum of the former serves as

TABLE I.  
*Action of Streptococci on Blood in Salt Solution and Bouillon.*

Strain.	Source.	Washed rabbit corpuscles. U S Army technique.	Hemolysis in 2 hrs. at 37°C.			
			Horse.	Human.	Rabbit.	Beef.
A-Cow 1	Milk from individual cow.	—	—	—	—	—
A-Cow 8	" " "	—	—	—	—	—
A-Cow 18	" " "	—	—	—	—	—
A-Cow 25	" " "	—	—	—	—	—
B-Cow 18	" " "	—	—	—	—	—
H-Cow 71	" " "	—	—	—	—	—
H-Cow 72	" " "	+	—	—	—	—
J-C10	" " "	—	—	—	—	—
J-C59	" " " (mastitis).	—	—	—	—	—
J-MJ	Milk pail.	—	—	—	—	—
J-E7	" from individual cow.	—	—	—	—	—
J-C65	Udder of cow at autopsy (mastitis).	+	±	—	—	—
Cheese 1	Cream cheese.	—	—	—	—	—
" 2	" "	—	—	—	±	—
B-Cow 2b	Milk from individual cow (mastitis).	++++	+++	++	—	—
H-Cow 108	Milk from individual cow (mastitis).	++++	++++	+++	++++	+++
H-M	Human throat.	+++	+++	—	—	—
X-32	" "	++++	+++	++	+++	+++
X-38	" lymph node.	++++	+++	++	+++	±
X-40	" spleen.	++++	+++	+++	++++	+++
X-43	" kidney.	+++	++++	++++	—	—
Imp. 1	Impetigo contagiosa.	+++	++++	—	—	—
" 2	" "	++++	+++	+++	—	—
D-AD4	Human throat.	++++	++++	+++	—	—
X-44	" empyema.	++++	++++	++	—	—
X-45	" "	++++	++++	++++	++++	++++
X-46	" "	++++	++++	++++	++++	++++
A-SH	" peritoneal cavity.	++++	++++	++++	—	—
Control.	Sterile salt solution.	—	—	—	—	—

Complete hemolysis is indicated by + + + + . Tubes were shaken each half hour.

medium for the streptococci. The importance of proper dilution and the influence of the length of period of incubation are shown in Table II. This table also illustrates strikingly the difference in hemolytic activity of bovine and human strains under these conditions.

TABLE II.  
*Action of Diluted Cultures on Defibrinated Blood. Effect of Dilution and Length of Incubation.*

Strain and dilution.	Incubation at 37°C.				Incubation at 37°C.				Strain and dilution	Incubation at 37°C.			
	1 hr.	1 hr.	1½ hrs.	2 hrs.	1 hr.	1 hr.	1½ hrs.	2 hrs.		In room over night.	In room over night.	1 hr.	1 hr.
A-Cow 1									B-Cow 2b				
1:5	-	-	-	+	+	+	+	+	1:5	-	+	+++	+++
1:10	-	-	-	-	-	-	-	-	1:10	-	+	+++	+++
1:20	-	-	-	-	-	-	-	-	1:20	-	+	+++	+++
1:40	-	-	-	-	-	-	-	-	1:40	-	-	+	++
1:80	-	-	-	-	-	-	-	-	1:80	-	-	-	-
A-Cow 8									H-Cow 108				
1:5	-	-	-	+	+	+	+	+	1:5	-	+	+++	+++
1:10	-	-	-	-	-	-	-	-	1:10	-	+	+++	+++
1:20	-	-	-	-	-	-	-	-	1:20	-	+	+++	+++
1:40	-	-	-	-	-	-	-	-	1:40	-	+	+++	+++
1:80	-	-	-	-	-	-	-	-	1:80	-	-	-	-
A-Cow 18									H-M				
1:5	-	-	-	-	-	-	-	-	1:5	-	+	+++	+++
1:10	-	-	-	-	-	-	-	-	1:10	-	+	+++	+++
1:20	-	-	-	-	-	-	-	-	1:20	-	+	+++	+++
1:40	-	-	-	-	-	-	-	-	1:40	-	-	-	-
1:80	-	-	-	-	-	-	-	-	1:80	-	-	-	-
A-Cow 25									X-32				
1:5	-	-	-	-	-	-	-	-	1:5	-	+	+++	+++
1:10	-	-	-	-	-	-	-	-	1:10	-	+	+++	+++
1:20	-	-	-	-	-	-	-	-	1:20	-	+	+++	+++
1:40	-	-	-	-	-	-	-	-	1:40	-	-	-	-
1:80	-	-	-	-	-	-	-	-	1:80	-	-	-	-

The tubes were shaken after each reading to keep the blood corpuscles from settling out.

TABLE II.—Concluded

Strain and dilution.	Incubation at 37°C.				Strain and dilution.	Incubation at 37°C.				In room over night.	In room over night.
	1 hr.	1 hr.	1½ hrs.	2 hrs.		1 hr.	1 hr.	1½ hrs.	2 hrs.		
J-C59	-	-	-	-	Imp. 2	++	++	++	++	++	++
1:5	-	-	-	-	1:5	++	++	++	++	++	++
1:10	-	-	-	-	1:10	+	++	++	++	++	++
1:20	-	-	-	-	1:20	-	++	++	++	++	++
1:40	-	-	-	-	1:40	-	+	++	++	++	++
1:80	-	-	-	-	1:80	-	-	+	++	++	++
J-MJ	-	-	-	-	D-AD4	++	++	++	++	++	++
1:5	-	-	-	-	1:5	++	++	++	++	++	++
1:10	-	-	-	-	1:10	+	++	++	++	++	++
1:20	-	-	-	-	1:20	+	++	++	++	++	++
1:40	-	-	-	-	1:40	-	+	++	++	++	++
1:80	-	-	-	-	1:80	-	-	-	-	-	-
J-E7	-	-	-	-	X-44	++	++	++	++	++	++
1:5	-	-	-	-	1:5	++	++	++	++	++	++
1:10	-	-	-	-	1:10	#	++	++	++	++	++
1:20	-	-	-	-	1:20	-	+	++	++	++	++
1:40	-	-	-	-	1:40	-	-	+	++	++	++
1:80	-	-	-	-	1:80	-	-	-	-	-	-
J-C65	-	-	-	-	X-45	++	++	++	++	++	++
1:5	-	-	-	-	1:5	++	++	++	++	++	++
1:10	-	-	-	-	1:10	+	++	++	++	++	++
1:20	-	-	-	-	1:20	+	++	++	++	++	++
1:40	-	-	-	-	1:40	+	++	++	++	++	++
1:80	-	-	-	-	1:80	#	++	++	++	++	++



*Appearance in the Blood Agar Plate.*

In the blood agar plate all the strains selected belong to the beta type. They show certain quantitative differences, however, as indicated in Table III.

TABLE III.  
*Hemolysis in Horse Blood Agar.*

Strain.	Character of the zone produced by the deep colony.
A-Cow 1	
A-Cow 8	
A-Cow 18*	
A-Cow 25	
B-Cow 18*	
H-Cow 71	
H-Cow 72	
J-C10	
J-C59	
J-MJ	
J-E7	
J-C65	
Cheese 1	
" 2	
B-Cow 2b*	
H-Cow 108	
H-M	
X-32	
X-38*	
X-40	
X-43	
Imp. 1	
" 2	
D-AD4*	
X-44	
X-45	
X-46	
A-SH	

Blood agar plates of these strains are illustrated in a previous article (1).

The zones of hemolysis produced by the pathogenic beta type hemolytic streptococci of human origin are of uniform size and character, but those produced by the bovine strains show considerable variety. It is, therefore, often possible on seeing a deep colony in

a blood agar plate for the first time to state with a fair degree of certainty that the organism is not of the human pathogenic variety. However, since some bovine streptococci produce zones much like those of human strains, it is not possible to state positively that such a colony is of human origin. Four such strains are indicated in Table III.

#### *Fermentation Reactions.*

In Table IV is given the titratable acidity after incubation for 1 week of cultures in fermented bouillon plus 5 per cent of sterile horse serum and 1 per cent of the test substance indicated.

TABLE IV.  
*Fermentation Reactions.*

Strain.	Titratable acid (per cent normal).					
	Saccharose.	Lactose.	Salicin.	Raffinose.	Inulin.	Mannite.
A-Cow 1.....	4.65	4.4	1.4	1.15	1.15	0.95
A-Cow 8.....	4.8	4.85	1.45	1.1	1.15	1.0
A-Cow 18.....	4.7	5.0	1.48	1.3	1.0	1.0
A-Cow 25.....	4.75	5.0	1.15	1.1	1.1	1.1
B-Cow 18.....	3.85	5.0	1.2	0.95	0.95	0.9
H-Cow 71.....	4.9	5.0	3.3	0.55	0.55	0.65
H-Cow 72.....	4.95	4.85	3.25	0.55	0.7	0.5
J-C10.....	5.3	4.8	4.4	0.6	0.55	0.3
J-C59.....	4.95	4.7	3.8	0.65	0.6	0.45
J-MJ.....	3.45	3.35	0.5	0.45	0.5	0.55
J-E7.....	4.75	4.6	3.7	0.5	0.55	0.5
J-C65.....	4.45	3.15	3.25	0.7	0.2	0.3
Cheese 1.....	5.7	4.7	5.0	0.8	0.35	3.8
“ 2.....	3.4	4.45	5.6	0.8	0.3	3.65
B-Cow 2b.....	3.6	3.65	4.8	1.05	0.9	0.85
H-Cow 108.....	3.6	3.2	3.2	1.0	1.0	0.8
H-M.....	3.55	3.7	3.55	0.8	0.6	0.85
X-32.....	3.55	3.55	4.7	0.9	0.75	0.95
X-38.....	3.95	3.65	4.8	0.8	0.85	0.8
X-40.....	3.85	3.15	4.9	0.8	0.8	0.7
X-43.....	3.9	3.25	3.4	1.05	1.0	1.0
Imp. 1.....	4.0	3.4	3.55	0.55	0.9	0.7
“ 2.....	2.9	3.5	2.95	0.6	0.5	0.35
D-AD4.....	3.25	2.85	2.65	0.85	0.85	0.7
X-44.....	3.7	3.45	3.35	0.4	0.75	0.7
X-45.....	3.9	2.8	2.5	0.35	0.25	0.3
X-46.....	3.6	3.75	3.8	0.35	0.1	1.5
A-SH.....	3.65	1.0	4.35	1.0	1.2	3.4

The titratable acidity of the medium was 0.5 to 1 per cent normal. A titratable acidity of less than 1.5 is regarded as a negative fermentation reaction.

*Limiting Hydrogen Ion Concentration.*

In Table V is given the hydrogen ion concentration of cultures in 1 per cent dextrose bouillon after incubation for 68 hours.

TABLE V.

*Limiting Hydrogen Ion Concentration in Dextrose Bouillon.*

Strain.	pH				Strain.	pH
	24 hrs.	68 hrs.	72 hrs.	116 hrs.		
A-Cow 1.....		4.6			B-Cow 2b.....	5.2
A-Cow 8.....		4.5			H-Cow 108.....	5.2
A-Cow 18.....		4.6			H-M.....	5.2
A-Cow 25.....		4.5			X-32.....	5.2
B-Cow 18.....		4.4			X-38.....	5.2
H-Cow 71.....		4.5			X-40.....	5.1
H-Cow 72.....		4.6			X-43.....	5.2
J-C10.....		4.6			Imp. 1.....	5.3
J-C59.....		4.5			" 2.....	5.3
J-MJ.....	5.8	5.3		5.3	D-AD4.....	5.3
J-E7.....	5.1	4.6	4.3		X-44.....	5.4
J-C65.....		5.1			X-45.....	5.2
Cheese 1.....		4.4			X-46.....	5.2
" 2.....		4.4			A-SH.....	5.2

Hydrogen ion determinations were made colorimetrically according to Clark and Lubs (Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191) with methyl red as an indicator.

The streptococci studied by Avery and Cullen (2) reached their limiting hydrogen ion concentration within 24 hours. This was also true of all the known human strains and most of the bovine strains studied by the author. However, a few of the latter did not; e.g., Strain J-E7. Neither could Strain J-MJ be depended upon in this respect. It was also noted that some substances, e.g. salicin, were fermented more slowly than others. The limiting hydrogen ion concentration was reached later in media containing this substance.

*Growth in Milk.*

Reference to Table VI shows that no sharp distinction between bovine and human strains can be drawn from their ability to coagulate milk in test-tubes. After incubation for 5 or 6 days most of the

strains, human and bovine, had coagulated the milk. In 24 hours only about half the bovine strains had done so. In 48 hours some of the human strains had partially coagulated the milk and some of the bovine strains had done no better. The most that can be said is that none of the strains of human origin caused coagulation of milk within 24 hours, whereas about 50 per cent of the bovine strains did so.

TABLE VI.  
*Coagulation of Milk.*

Strain.	Period of incubation.			Strain.	Period of incubation.		
	24 hrs.	48 hrs.	6 days.		24 hrs.	48 hrs.	6 days.
A-Cow 1.....	++	++++	++++	B-Cow 2b....	-	-	++++
A-Cow 8.....	++	++++	++++	H-Cow 108...	-	++	++++
A-Cow 18.....	+++	++++	++++	H-M.....	-	++	++++
A-Cow 25.....	+++	++++	++++	X-32.....	-	-	+++
B-Cow 18.....	+++	++++	++++	X-38.....	-	+	+++
H-Cow 71.....	++	++++	++++	X-40.....	-	-	=*
H-Cow 72.....	+++	++++	++++	X-43.....	-	-	+++
J-C10.....	-	+	++++	Imp. 1.....	-	+	+++
J-C59.....	-	+	++++	" 2.....	-	-	=*
J-MJ.....	-	-	-*	D-AD4.....	-	-	=*
J-E7.....	+	++	++++	X-44.....	-	++	++++
J-C65.....	-	-	-*	X-45.....	-	+	++++
Cheese 1.....	-	+	++++	X-46.....	-	+	++++
" 2.....	++++	++++	++++	A-SH.....	-	-	-†

\* Coagulated when placed in boiling water.

† Not coagulated when placed in boiling water.

++++ indicates complete coagulation.

#### *Reaction to Methylene Blue.*

It has been claimed by Sherman and Albus (4) that cultures of *Streptococcus lacticus* reduce methylene blue in milk while those of *Streptococcus pyogenes* fail to do so and in fact fail to grow in this medium. Their results are striking, though we doubt the validity of their method of selecting cultures. We cannot agree in applying the name *Streptococcus pyogenes* to all streptococci isolated directly from the udder. The strains studied above were inoculated into methylene blue milk (1: 20,000) according to the technique described by Sherman

and Albus. The two strains from cheese reduced the methylene blue almost completely in less than 16 hours, but certain strains from the udder and from human patients also produced partial reduction in less than 24 hours, others within 66 hours, and others not at all. On methylene blue agar plates (1: 20,000) streaks of Strains Cheese 1 and Cheese 2 grew luxuriantly, while none of the other strains grew at all. It is interesting to note, however, that streptococci which were inhibited by methylene blue in the presence of oxygen grew well in media containing methylene blue which was reduced by some other agent. This was demonstrated by boiling tubes of methylene blue agar in a water bath until the dye was colorless, inoculating the agar in fluid condition, chilling it quickly, and incubating. After several hours the blue color returned to the upper half inch of the medium and in this portion no colonies developed. Below the upper half inch the agar remained colorless and here the streptococcus colonies grew as well as in agar containing no methylene blue.

#### CONCLUSION.

None of the procedures described serves by itself to differentiate streptococci of human and bovine origin with certainty, though each of them serves as a strong presumptive test. Most strains fall easily into the human or bovine group by all the tests. Eliminating these from consideration we have left certain irregular strains listed in Table VII.

TABLE VII.

#### *Irregular Strains.*

Strain.	Source.	Origin as indicated by.		
		Blood agar plate.	Blood-salt solution.	Acidity in dextrose bouillon.
J-E7	Normal udder.	Human (doubtful).	Bovine.	Bovine.
Cheese 1	Cream cheese.	" "	"	"
" 2	" "	" "	"	"
J-C65	Diseased udder.	" "	"	Human.
J-MJ	Milk pail.	Bovine.	"	"

Taking all characters into consideration we are inclined to regard Strains J-E7, Cheese 1, and Cheese 2 as undoubtedly of bovine origin. Strain J-MJ also is representative of a group of streptococci which Jones<sup>2</sup> has found in milk and which is being further studied by him. There remains Strain J-C65 which for the present must be regarded as of doubtful origin.

The author is indebted to Dr. F. S. Jones for the strains of streptococci lettered J, and to Miss Marion L. Orcutt for valuable assistance.

#### BIBLIOGRAPHY.

1. Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1919.
2. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.
3. Methods for the isolation and identification of *Streptococcus haemolyticus*, adopted by the Medical Department of the United States Army, New York, June 1, 1918.
4. Sherman, J. M., and Albus, W. R., *J. Bacteriol.*, 1918, iii, 153.

---

<sup>2</sup>Jones, F. S., personal communication.



## DAIRY INFECTION WITH STREPTOCOCCUS EPIDEMICUS.

By J. HOWARD BROWN, PH.D., AND MARION L. ORCUTT.

(*From the Department of Comparative Pathology of Harvard Medical School,  
and the Department of Animal Pathology of The Rockefeller  
Institute for Medical Research, Princeton, N. J.*)

### PLATE 9.

(Received for publication, October 15, 1919.)

The milk-borne streptococcus epidemic about to be reported is of interest not because of any unusual features or because of any intensity of the epidemic, but because of the definiteness of the findings and the promptness with which they were obtained. There were also certain unusual clinical features.

The dairy involved was one producing a high grade of raw milk for sale in Boston. In February, 1917, there was prevalent, especially among children, a malady which was usually diagnosed as epidemic adenitis, more rarely as sore throat, pharyngitis, or tonsillitis. The initial symptom was sore throat of moderate severity, rarely such as to be diagnosed as septic sore throat, a reportable disease in Massachusetts. A few patients experienced a peculiarly nauseating nasopharyngitis. A few cases of otitis media occurred, but the most constant development was inflammation of the submaxillary and cervical lymph nodes. Recovery was sometimes quite slow and characterized by recurrent periods of high temperature.

At the time the authors were consulted by the dairy several pediatricians had noticed that among their patients an undue proportion were consumers of this milk. On the other hand, an infants' hospital supplied with milk by this dairy had reported no cases of the disease.

On the afternoon of February 23, a conference was called by the officials of the dairy. Physicians whose patients were using this milk were invited to attend this conference and also the authors. Although there was no conclusive evidence that the milk was at fault, a complete investigation was undertaken at once. The following measures were adopted. (1) At the conference physicians were sup-

plied with sterile swabs for the collection of pathologic material from patients. (2) Arrangements were made to obtain samples of milk from individual cows of the dairy herd beginning the next morning. (3) Swabs from the throats of employees of the dairy were to be examined as soon as the milk had been studied. (4) All the milk of the dairy was to be pasteurized in the final bottles for the time being.

Apparently the epidemic among consumers of the milk ended immediately. The initial symptoms of the last case reported were noticed on February 24.

Since in a paper by Dr. Theobald Smith and the author (1) the milk-borne epidemics described were designated A to G inclusive, this one and the dairy involved will be referred to as Epidemic H and Dairy H.

#### *The Human Material.*

Swabs received were soaked for 15 minutes in about 1 cc. of sterile salt solution which was pipetted directly into the tube in which the swab was received. The salt solution was then diluted, added to fluid blood agar (standard meat infusion agar, 12 cc., plus defibrinated horse blood,  $\frac{2}{3}$  to 1 cc., at 45°C.), and immediately poured into a Petri dish. After the moisture condensing inside the dish had been allowed to evaporate the plate was incubated at 37°C. in the inverted position.

Fifteen swabs were received from patients using milk of Dairy H. Of these, twelve were from throats or tonsils and three from ears. In the blood agar plates of twelve of these swabs, including those from ears, was found the organism which we have learned to recognize as *Streptococcus epidemicus* Davis.<sup>1</sup> The appearance of this organism in the blood agar plate is such as to make it readily recognizable (2). It produces the beta type of hemolysis. The surface colonies are large and watery, round when isolated but confluent and amebiform in the streak. Organisms in the fresh surface colonies are encapsulated. This streptococcus was present in large numbers on most of

<sup>1</sup> The name *Streptococcus epidemicus* is used tentatively. The organism is so nearly like *Streptococcus pyogenes* that it may be regarded as a well defined variety of the latter rather than as a true species, and if so should be called *Streptococcus pyogenes* (var. *epidemicus*).

the swabs; in pure culture on three of them, two from throats and one from an ear, and in almost pure culture on several others. It was not found on three of the swabs from patients using Dairy H milk. One of these was a convalescent patient and the material from the other two was not properly diluted and the plates were poor. These should have been done over again, but in view of the evidence already at hand and because of the great quantity of material from cows waiting for study at the time, this was not done.

Swabs were studied from four adults in families where children had the infection. The children in these families used milk from Dairy H but the adults did not use it regularly. A diagnosis of epidemic adenitis was not made in the case of the adults but they complained of coughs, colds, or sore throat. In none of them was *Streptococcus epidemicus* found.

Nine swabs were studied from patients not using milk from Dairy H. Three of these were from cases with a clinical diagnosis of epidemic adenitis. The others were diagnosed as sore throat or tonsillitis. From none was *Streptococcus epidemicus* isolated. Other pathogenic streptococci were recovered from some of them.

Most of the patients from whom *Streptococcus epidemicus* was isolated had developed initial symptoms on February 13 to 18. Most of the swabs were taken on February 24, 25, and 26. The last case reported developed initial symptoms on February 24, and the swab was taken on March 2.

#### *The Dairy Herd.*

All the milk sold by Dairy H was produced by its own herd, about 112 cows being milked at this time. The milk was produced and handled under exceptionally good conditions. Precautions were taken to exclude the milk from cows with garget. The herdsman drew the fore-milk from each teat onto a fine wire screen in order to detect the presence of flocculi or viscosity. The so called gang system of milking was in use, each man milking the next cow in line at the head of the gang of milkers as he finished milking the last one. The milk was quickly cooled and bottled at the farm.

The herd was housed in three barns, A, B, and C. On the morning of February 24 samples of milk were taken from twenty cows in Barn A. A stream of milk from each of the four quarters of every cow was milked directly into a sterile cream bottle. The samples were packed in ice and taken directly to the laboratory where they were centrifuged and the sediment was plated out in horse blood ager. A stained film of the sediment was also made. In making the blood agar plates it was found useful to employ both deep and surface streak inoculation of the same plate. The tube of blood ager was first inoculated in fluid condition, then after the plate had been poured and hardened it was streaked in three or four places. This gave an opportunity to observe both deep colonies and surface streaks on the same plate.

It happened that one of the first samples yielded in pure culture large numbers of *Streptococcus epidemicus* resembling in every way the organisms isolated from the patients (Fig. 1). The stained film of the milk sediment revealed large numbers of leucocytes and many short chains and pairs of round or flattened cocci. The film alone would have attracted suspicion but could not have been relied upon for a diagnosis. The milk of this cow (No. 108) had not yet roused the suspicion of the herdsman or milkers and was being used. As soon as this culture was discovered the cow was isolated on February 25, and on the morning of February 26 samples of milk were taken from each quarter of the udder. By this time the sample from the left fore quarter was noticeably thick and yellow, while samples from the other three quarters were normal in appearance. Cultures showed the left fore quarter only to be infected.

By February 26 samples of milk from all the cows of the herd had been similarly cultured and examined. None revealed any streptococci resembling those from the patients and Cow 108. Among these cows, however, were three known to have garget in one quarter of the udder. The milk of this quarter was regularly discarded. We studied samples of milk from these quarters culturally and found streptococci which produced more or less hemolysis in the blood agar plate but did not closely resemble the streptococci isolated from the patients and Cow 108.

The location of Barn A and the method of handling the milk at the farm offer a suggestion as to why the epidemic was not more widespread among the patrons of the dairy. They also serve to explain why there was not a single case at an infants' hospital where the milk was used. At the barns the milk was poured from the milking pails into large sterile cans and carried by wagon to the milk house where it was poured from the cans into the mixing tank. From the mixing tank it ran through the cooler and was bottled, the bottling going on continuously while fresh milk was being poured into the mixing tank. The apparatus was properly sterilized before each milking and the whole process carried on under very good conditions. There might be in the mixing tank the milk from not more than ten or fifteen cows at any one time. Barns B and C were within about 200 yards of the milk house and milk from these barns was therefore the first to enter the mixing tank. Milk from Barn A which was a quarter mile or more from the milk house was likely to enter the mixing tank toward the latter part of the milking. Hence, the larger part of the milk escaped contamination by the milk of Cow 108 which was in Barn A.

#### *The Dairy Employees.*

The theory advanced by Smith and Brown (1) that epidemic milk-borne tonsillitis is due not to the streptococci of ordinary bovine mastitis but to streptococci of human origin inoculated into the milk ducts of cows during milking has now received considerable support from the work of other authors.

In view of the recent remarks of Keegan (3) it seems necessary again to emphasize the fact that infection of the cow's udder by *Streptococcus epidemicus* may persist for some time without gross evidence of mastitis. That such an infection is not common is shown by the work of Jones (4) who studied over 80 cases of mastitis in cows without finding *Streptococcus epidemicus* except possibly in one instance. These facts make the finding of this organism in the udder or milk supply in association with an epidemic all the more significant. It was not the intention of Smith and Brown to deny the possibility of contact infection.

The theory of Smith and Brown offers a plausible explanation of the relative rarity of such milk-borne epidemics notwithstanding the wide prevalence of bovine mastitis, or garget, and its occurrence in

TABLE I.  
*Infected Individuals at the Dairy Farm.*

Individual.	Employment and habitat	Cultural history	Clinical history.
W	Driver of automobile truck and miscellaneous work. Lived at farm boarding house.	<i>S. epidemicus</i> + Feb. 26, Mar. 20, Apr. 2, 9. Never present in large numbers. Negative cultures at intervals between above dates.	One tonsil large but no clinical symptoms at any time. Quinsy sore throat shortly before Feb. 26. No clinical symptoms after Feb. 26.
C	Milker. Lived at farm boarding house.	<i>S. epidemicus</i> + Feb. 26, 28, Mar. 1, 2, 5, 8, 10, 12, 13, 14. Often in considerable numbers. Negative cultures Mar. 15, 16, 17, 20, and thereafter.	Mar. 12 to 17. Daily irrigation of crypts of tonsils with hydrogen peroxide.
-S	Milker and feed mixer. Lived at home. Father of K.	<i>S. epidemicus</i> + Mar. 2, 4, 5, 6, 8, 10, 12, 13, 14, 16. Often present in fair numbers. Negative cultures Mar. 17, 20, Apr. 2, and thereafter.	Large tonsils with large crypts but no clinical symptoms before Mar. 15; Mar. 12 to 17. Daily irrigation of crypts of tonsils with hydrogen peroxide.
M	Milker. Lived at farm boarding house.	<i>S. epidemicus</i> + Mar. 2, 5, 6, Apr. 23, 30, May 14, 29, June 5, 11. At first present in considerable numbers; later very few. Negative cultures Feb. 26, 28, Mar. 3, 8, 10, 20, Apr. 2, May 7, 25.	Mar. 15. Complained of rheumatism. Apr. 2. Confined to home for several days with rheumatism of back. No throat symptoms. Mar. 10. Complained of rheumatism. " " 20. Confined to bed. Temperature. Left knee swollen and painful. Sent to hospital. Apr. 2. Back from hospital. Re-

D	Milker. Lived at farm boarding house.	<i>S. epidemicus</i> + Mar. 8, May 14. Present in small numbers. Negative cultures Mar. 3, 5, 14, 20, Apr. 16.	Left tonsil enlarged. Mar. 15. Complained of rheumatism. " 20 to Apr. 16. Unable to work at times. Puffy swelling of wrist and ankle. Apr. 24. Part of left tonsil cut off by doctor.	Throat apparently normal. No clinical symptoms at any time.	Had been quarantined at home with "diphtheria" for several weeks pre- vious to Mar. 23. No swabs exam- ined during that time. No subsequent clinical symptoms.	Said to have had several attacks of quinsy sore throat during past few months.	The swab was taken just previous to removal of tonsils on Apr. 30. No other swabs taken.
K	Milker. Lived at home. Son of S.	<i>S. epidemicus</i> + Mar. 20, 23, 26. Present in fair numbers. Negative cultures repeatedly before and after above dates.					
J	Milker. Lived at home.	<i>S. epidemicus</i> + Mar. 23, Apr. 2. Present in small numbers. Negative cultures Mar. 26 and after Apr. 2. No diphtheria bacilli found.					
Miss V.	Father and mother employed on farm. Lived at home.	<i>S. epidemicus</i> + Apr. 30. Present in moderate numbers.					

practically all dairy herds of any size. With this theory in mind our next effort was to determine the source of the infection of Cow 108. Unfortunately two of the milkers who had attended this cow at the time the epidemic started had left the employ of the dairy and it was impossible to obtain material or clinical data from them. Under the system of milking in use it was impossible to connect the act of milking any given cow with any particular milker.

On February 26 swabs were taken from the throats of fifteen employees, mostly milkers, at the dairy farm. All these men reported themselves in good health. One milker, C, had recently recovered from quinsy sore throat, but this man was said not to have worked in Barn A where Cow 108 was kept. *Streptococcus epidemicus* was found on swabs from C and another man, W, who drove the milk truck and did other odd jobs about the farm but did no milking. Both C and W lived at the farm dormitory and boarding house. Cultures from the other men were negative on this date. Swabs were taken and cultured from as many of the men as possible every few days. In Table I is given a tabular summary of the study of infected employees listed in the order of the discovery of the infection. Special attention is called to J, a milker, living at home, who had just returned to work after being under treatment for diphtheria for several weeks. Two women were employed at the farm boarding house. Swabs from both of these were examined repeatedly with negative results. One, Mrs. H., reported that she had "lost her voice several weeks ago as a result of laryngitis," but appeared well at the time of this investigation. The other, Mrs. V., had no clinical history. Her husband also was employed on the farm but gave no clinical history, and no positive cultures were obtained from him. On April 30, however, it was learned that a young daughter of Mr. and Mrs. V. was to be taken to Boston to have her tonsils removed. It was learned that this child had suffered from several attacks of quinsy sore throat during the past few months. She did not live on the farm but a swab from her throat was obtained previous to operation. Moderate numbers of *Streptococcus epidemicus* were found. It was also learned from K on March 26 that he had a young sister who "came near having pneumonia lately" and another sister who had frequent "earache due to adenoids." Swabs from these people could not be obtained.

Altogether swabs were studied, in most cases at regular intervals of a few days, from twenty-six persons connected with the dairy. From eight of these *Streptococcus epidemicus* was isolated at one time or another. At least two harbored the organism when the first swabs were taken on February 26.

The incidence of rheumatism among the infected men is noteworthy. There was no complaint of rheumatism among the non-infected employees.

From the data it seems impossible to connect any of these individuals with the introduction of the infection into the dairy and the infection of Cow 108. There are too many possibilities. It may be that C, or J, some member of the family of S, or Miss V. was the source of infection, or it may have been one of the men who left the dairy before the investigation was started. On the other hand, it seems likely that some of these and perhaps other persons were infected by using the milk of Cow 108, since all the employees and their families used the milk of the dairy. It is also possible that there was a good deal of contact infection, and there seems no other way to account for the infection of K, D, and possibly S, M, and J after Cow 108 had been removed. It is also possible that J's disease was a pseudodiphtheria due to *Streptococcus epidemicus*.

After Cow 108 had been eliminated from the herd frequent examinations of the milk and of swabs from the men's throats were made. All infected men were kept from milking or handling the milk in any way.

#### *Data Bearing on the Mastitis of Cow 108.*

Cow 108 gave birth to a calf in November, 1916. She was apparently normal and gave a good quantity of milk from each quarter of the udder up to the time of this investigation on February 24, 1917. On this date, as related above, *Streptococcus epidemicus* was found in the mixed milk from the four quarters of the udder. A photograph of the blood agar plate made at this time is shown in Fig. 1. Samples of milk from individual quarters of the udder showed infection in only the left fore quarter. On February 26 the milk from this quarter was noticeably thick and yellow. *Streptococcus epidemicus* was present in pure culture. Samples of milk from individual quarters were

again studied on March 11. By this time the left fore quarter was manifestly shrunken, the milk from it diminishing in amount and being quite thick and yellow. The culture was as before. Milk from

TABLE II.  
Infected Cow 108.

Feb. 24. Mixed sample of milk from all four quarters. Gross appearance normal. Large numbers of *Streptococcus epidemicus* per cc. of milk. Cow isolated.

Date.	Milk from left fore quarter.		Milk from left hind quarter.	
	Gross appearance of milk.	<i>S. epidemicus</i> per cc.	Gross appearance of milk.	<i>S. epidemicus</i> per cc.
<i>1917</i>				
Feb. 25	Thick; yellowish.	Large numbers.	Normal.	None.
Mar. 11	" yellow.	" "	"	30,000±
" 15			"	15,000
" 15-20	Left hind teat injured, probably by being stepped on.			
" 20			Curdled; yellowish.	872,000
" 25	Cow removed to animal hospital.			
" 26	Serous; yellow. 75 cc.	15,000	Normal. 275 cc.	375,000
" 28	Watery. Few cc.	39,300	" 375 "	200,000
Apr. 1	Serous; flocculent. Few cc.	22,000	"	23,000
" 8	Serous. Few cc.	175,000	" 250 cc.	28,500
" 15	" with white stringy particles.	32,000	"	57,000
" 23	Serous; stringy. 10 cc.	90,000	14 million leucocytes per cc.	
			Slightly flocculent.	75,000
			250 cc.	
			16 million leucocytes per cc.	
May 2	Serous.	665,000	Normal.	32,000
" 9	" stringy. 5 cc.	57,500	" 250 cc.	29,500
" 16	Serous; stringy. 8 cc.	2,085,000	Slightly stringy. 250 cc.	60,000
June 6	Serous; thick yellow masses. 5 cc.	250,000,000	Normal. 150 cc.	50,000
" 20	Serous; thick yellow masses. 5 cc.	270,000,000	5 million leucocytes per cc.	
			Slightly stringy. 150 cc.	2,000

the other three quarters was quite normal in appearance and amount. In the plate of milk from the left hind quarter, however, were found 30,000 colonies of *Streptococcus epidemicus* per cc. of milk. On gross

examination this quarter of the udder was apparently normal. On March 15 were found about 15,000 of the streptococci per cc. of milk from the left hind quarter. Some time between this date and March 20 the teat of the left hind quarter was injured. When examined on March 20 by one of us the teat was swollen and blood was dripping from it occasionally. There was a cut leading outward from the meatus. The milk collected at this time was thick, yellow, curdled, and slightly tinted with blood. Culture revealed 872,000 of the infecting organism in apparently pure culture per cc. of milk. Soon after this the cow was removed from the farm to the Angell Memorial Animal Hospital<sup>2</sup> where it was accessible for study from the laboratory at Harvard Medical School. To avoid transference of the infection from infected to normal quarters by the hands of the milker, he was instructed always to milk the normal quarters first. The milk from the four quarters was studied for many weeks. During this time the general condition of the cow was normal and the right fore and hind quarters of the udder remained uninfected. The results of the examination of the milk from the left fore and hind quarters are shown in Table II.

The point of greatest interest in as far as the epidemic is concerned is that there was detected bacteriologically an infection with *Streptococcus epidemicus* of the left fore quarter of the udder of this cow before it had attracted the attention of the herdsman and milkers at the dairy, that it was detected in the left hind quarter a week or more before the milk showed any gross change, and that the milk of this quarter again returned to almost normal appearance though the infection persisted for many weeks.

At the end of June the authors left Boston. Further study of Cow 108 was carried on by Dr. E. E. Tyzzer and Dr. Marshal Fabyan.

#### *Study of Cultures.*

Although *Streptococcus epidemicus* was isolated 80 times from patients, dairy employees, and Cow 108, all the strains were indistinguishable from one another. A general description therefore suffices for all.

<sup>2</sup> The cow was kept in this hospital through the courtesy of Dr. S. J. Mixter of Boston.

*Appearance in Blood Agar.*—In the blood agar plate composed of standard beef infusion agar plus 5 to 10 per cent of horse blood, the deep colonies after 18 to 24 hours incubation are biconvex, usually a little larger than those of typical *Streptococcus pyogenes*, and surrounded by a distinct clear colorless zone of hemolysis about 2 to 2.5 mm. in diameter unless the plate is crowded with colonies. There are no intact corpuscles next to the colony and no discoloration. The appearance is that of the beta type. The hemolyzed zone of the deep colony is essentially like that of *Streptococcus pyogenes*, but it may be a little slower in developing. The surface colonies on the blood agar plate or slant serve to distinguish this organism from *Streptococcus pyogenes*. They are large colonies, 1 to 4 or 5 mm. in diameter, watery when grown in a humid atmosphere but drying down rapidly to thin transparent films when exposed to a dry atmosphere; e.g., that of the room. If the surface colonies are close together, as in a streak, they become confluent and amebiform. Hemolysis appears more slowly about the surface colonies than about the deep ones, and in fact may not be very noticeable after incubation over night. This is partly due to the rapid overgrowth of the surface colony, obscuring the zone of hemolysis beneath. Not infrequently the surface colonies at this stage have a greenish tint which, however, is not due to the methemoglobinization of underlying blood corpuscles, though it may possibly be due to the formation of methemoglobin from released hemoglobin which has diffused into the substance of the colony. It is easily distinguished from the alpha type of appearance produced by pneumococci and *viridans streptococci*. The surface colony itself is like that of *Streptococcus (Pneumococcus) mucosus*, the Type III pneumococcus of Cole, but the latter organism produces the alpha appearance in blood agar and is further distinguished by fermentation and immunological reactions.

*Morphology.*—If some of the growth from a fresh watery surface colony is examined microscopically there are found diplococci and short chains of streptococci with large capsules enveloping the entire group. These capsules are best seen by suspending the material in a droplet of bouillon and a suitable India ink, covering with a cover-slip, and examining the moist preparation under the microscope. They are also revealed by the Huntoon capsule stain. The cocci themselves

are round or slightly flattened, closely packed together within the chain. They are Gram-positive and when seen encapsulated closely resemble *Streptococcus (Pneumococcus) mucosus*.

*Appearance in Bouillon.*—In bouillon there is nothing to distinguish *Streptococcus epidemicus* from *Streptococcus pyogenes*, though the former is likely to produce more clouding and less sediment than the latter. Usually the bouillon is fairly well clouded and there is a moderate amount of finely flocculent sediment which is easily disintegrated and suspended by shaking. Microscopically there are found moderately long chains of streptococci. A small amount of capsular substance may or may not be present.

*Fermentation Reactions.*—The fermentation reactions of all the strains were determined after incubation for 1 week in large test-tubes by titration of the total acidity of cultures in fermented bouillon plus 5 per cent of sterile horse serum and 1 per cent of the test substance.

In Table III the titratable acidity of representative strains is expressed as per cent normal acid. No subtraction has been made for the reaction of the medium. The fermentation reactions are the same as those of *Streptococcus pyogenes*. All the strains fermented saccharose, lactose, and salicin, but not raffinose, inulin, or mannite. It is to be assumed that they ferment dextrose and maltose also.

*Comparison with Bovine Strains.*—For comparison with the other strains there are included in Table III two strains of bovine hemolytic streptococci, H-Cow 71 and H-Cow 72. They produce a higher titratable acidity in saccharose and lactose media than do the strains of *Streptococcus epidemicus*. These two strains came from the milk of apparently normal cows. In blood agar they produce the beta type of hemolysis, but the zones show minor differences from those of the other strains. The zones of hemolysis develop rather slowly, those of H-Cow 71 remaining small, and those of H-Cow 72 becoming broad but with a hazy outer portion. Neither strain produces capsules. The individual elements are large. These minor differences between such non-pathogenic bovine strains and pathogenic hemolytic streptococci of human origin may easily escape the attention of one without considerable experience in the study of streptococci, but they are convincing when recognized. In 1915 it was said that:

"The success likely to attend the tracing of such epidemics to their source will depend upon a minute, detailed study of individual strains of streptococci and the discovery of certain minor distinguishing characteristics as guides" (1). This is still true and something

TABLE III.  
*Fermentation Reactions.*

Strains from.	Titratable acid (per cent normal).					
	Saccharose.	Lactose.	Salicin.	Raffinose.	Inulin.	Mannite.
<b>Patients.</b>						
H-3	3.9	3.15	2.65	0.9	1.05	1.05
H-10	3.55	3.1	3.0	0.95	1.1	1.1
H-11	3.4	2.9	2.7	0.4	0.3	0.3
H-14	3.6	3.3	2.55	0.6	0.65	0.3
H-16	3.6	3.1	2.5	0.6	0.7	0.6
H-17	3.6	3.0	2.85	0.6	0.7	0.85
H-18	4.1	3.05	2.55	0.3	0.55	0.2
H-20	4.05	2.9	2.7	0.7	0.5	0.75
H-21	3.15	2.9	2.65	0.6	0.85	0.45
H-23	4.05	2.95	2.7	0.35	0.55	0.5
H-40	3.7	3.3	2.4	1.0	1.15	1.1
<b>Employees.</b>						
H-W	3.8	3.15	3.25	0.9	1.2	0.9
H-C	3.6	3.2	2.6	0.95	1.05	1.1
H-S	3.65	3.0	3.2	0.85	0.6	0.9
H-K	3.7	3.25	3.3	0.8	0.65	0.85
H-M	3.55	3.7	3.55	0.8	0.6	0.85
H-D	3.75	4.1	3.3	0.9	0.6	Lost.
H-J	3.6	3.0	2.55	0.6	0.5	0.4
<b>Dairy cows.</b>						
H-Cow 108	3.6	3.2	3.2	1.0	1.0	0.8
H-Cow 71	4.9	5.0	3.3	0.55	0.55	0.65
H-Cow 72	4.95	4.85	3.25	0.55	0.7	0.5

The titratable acidity of the medium was 0.5 to 1 per cent normal. A titratable acidity of less than 1.5 is regarded as a negative fermentation reaction.

further has been done to facilitate the recognition of these minor distinguishing characteristics. Ayers and his associates have called attention to the differences in final hydrogen ion concentration which serve to distinguish streptococci from different sources and many pathogenic from non-pathogenic streptococci (5, 6). Avery and

Cullen (7) have reported the usefulness of the determination of hydrogen ion concentration in the differentiation of hemolytic streptococci of human and bovine origin. They found that in dextrose bouillon "the human type of *Streptococcus haemolyticus* reaches a final hydrogen ion concentration of pH 5.2 to 5.0, and the bovine type of pH 4.5 to 4.3." Among the strains of streptococci which they studied were the three strains from dairy cows listed in Table III. Their results for these strains were as follows:

Designation of strain.		pH	Diagnosis.
Brown and Orcutt.	Avery and Cullen.		
H-Cow 108	V 10	5.1	Human type.
H-Cow 71	V 8	4.5	Bovine "
H-Cow 72	V 9	4.5	" "

The fact that streptococci of bovine origin produce in carbohydrate media more acid than do pathogenic streptococci of human origin has been noted by various authors (Broadhurst (8), Stowell, Hilliard, and Schlesinger (9), and Smith and Brown (1)) employing the titration method, and has been utilized as a means of differentiating streptococci from these two sources. In our experience more than 90 per cent of bovine streptococci of the beta type produce in dextrose bouillon from 1 to 1.5 per cent more normal acid than do human streptococci of the beta type, and the method of titrating the total acidity against 0.05 N sodium hydroxide with phenolphthalein as an indicator is reliable in the hands of an individual worker using a medium of fairly constant composition for the comparative study of strains from both sources.

*Blood-Salt Solution Test.*—In another article (10) is described the behavior of strains from this epidemic in a suspension of blood in salt solution. It is sufficient here to call attention to the fact that also by means of this test Strains H-Cow 71 and H-Cow 72 fall into the bovine group while Strains H-Cow 108 and H-M fall into the human group.

*Animal Experiments.*—Soon after isolation 1 cc. of bouillon culture of a number of strains from representative sources was injected intravenously into rabbits, with results as indicated in Table IV.

The organism was not particularly virulent as judged by the mortality of rabbits injected. The most conspicuous lesions produced were those of the external ears and testes. The lesions of the ears appeared on the 4th to the 9th day and resembled erysipelas, sometimes affecting the whole pinna simultaneously, but more often

TABLE IV.  
*Inoculated Rabbits.*

Rab-bit.	Sex.	Culture.	Maximum temperature.	Weight (variation).	Localizations.	Result.
A	M.	H-10	105.5 (4th day).*	1,220-1,610	Apparently well throughout.	
B	"	H-C	107.3 (2nd day).	1,310-1,070	Both ears, 9th day. Left testis, 10th day.	Recovered (40 days).
C	"	H-S	105.1 (3rd day).	1,270-980	Right ear, 9th day. Lame in left hind leg temporarily, 10th day.	Recovered (40 days).
D	F.	H-M	106.0 (2nd day).	2,090-1,770	Endocarditis.	Died (4th day).
E	M.	H-M	106.6 (2nd day).	2,180-1,700	Both ears, 5th day. Both testes.	Very sick. Chloro-formed (7th day).
F	"	H-Cow 108	106.2 (2nd day).	1,330-1,150	Both ears, 4th day. Left eye, 4th " " testis, 4th day. Right testis, 14th day. Right hind foot, 14th day.	Died (29th day).
G	"	H-Cow 108 (19 mos. later).	106.5 (3rd day).	1,325-1,140	Left ear, 5th day. Right fore foot, 8th day.	Recovered (17 days).

\* The day on which the injection was made is counted as the 1st day.

starting in one area and migrating to the remaining parts. The ear became red, hot, considerably swollen, and heavy so that it drooped. In a day or two there appeared little droplets of blood-stained serum exuding at points on either surface of the ear. When cultured in blood agar plates these droplets were found to contain large numbers

of the streptococcus injected. There often appeared areas of purpura, blisters or bullæ, and occasionally a necrosis, dry gangrene, and sloughing off of a part of the ear. If the rabbit lived the inflammation gradually subsided after a week or more, the skin became dry, underwent desquamation, and finally the ear regained its normal appearance. A section of an ear from Rabbit E revealed the following changes.

The cartilage and narrow strip of dense fibrous tissue on either side of it are intact. The looser vascular connective tissue and subcutis are greatly distended and in large areas obliterated by a serofibrinous exudate containing much cell debris (necrotic connective tissue and degenerating cell nuclei) but very few leucocytes and not many endothelial cells. In the tissue on the dorsum of the ear the blood vessels contain plugs of fibrin and some are plugged with streptococci, but most of the streptococci are massed in the lymph spaces and tissue spaces surrounding blood vessels. A few streptococci are seen scattered in the tissue spaces just beneath the cutis. The tissue on the ventral surface of the ear is less vascular and here the streptococci are seen quite generally scattered about throughout the distended connective tissue and fibrinous exudate. In the section studied the epidermis was intact and there were no blisters or bullæ.

It is to be noted that in three out of five rabbits both ears showed similar lesions, whereas the injection was made into the vein of only one ear. In no case did the infection appear to spread from the site of injection.

The next most common lesion was orchitis which occurred in three of five male rabbits injected with freshly isolated cultures. The scrotum enclosing the testis became hot, purplish red in color, swollen, and tense, the testis remaining in the sac. In Rabbit B the process subsided after a few days and there was a desquamation of the skin of the scrotum. In Rabbit F the orchitis persisted until death and at autopsy both testes were found adherent to the scrotum. The left testis which had been longest diseased was simply a homogeneous yellow caseous mass. The right one was a sac of fluid yellow pus. Cultures from the right testis revealed *Streptococcus epidemicus* in pure culture. In Rabbit E the orchitis was not detected until the animal was chloroformed on the 7th day. Both testes were freely movable from scrotum to abdomen but had a mottled appearance more noticeable in the left testis. This testis was sectioned and showed the following changes.

Transverse section of the testis shows on one side a distinct area of necrosis involving the tubules and interstitial tissue, and extending at one point into the tunica vasculosa. In the center of this area the outlines of the tubules are distinct and the parenchymatous cells are distinguishable but structureless and without nuclei. At the periphery of the necrotic area the appearance is that of coagulation necrosis with numerous disintegrating nuclei. Polymorphonuclear cells are scattered throughout most of the tunica vasculosa. On the opposite side of the testis from the necrotic area is a mass of fibrin containing in its meshes large numbers of polymorphonuclear leucocytes and large mononuclear cells. This mass is apparently outside the tunica vasculosa but was probably inside the tunica albuginea which has been stripped away from most of the testis and is not present in the section though remnants of it are seen. On one portion of the epididymis a fibrinous exudate between the tunica vasculosa and tunica albuginea is plainly seen, while scattered through the connective tissue of the epididymis are many polymorphonuclear cells. The rete testis and the ducts of the epididymis are apparently normal as is also the connective tissue of the septula and that between the tubules of the testis. A few streptococci are seen within leucocytes.

Other lesions encountered and studied were an endocarditis in Rabbit D and a keratitis and conjunctivitis in one eye of Rabbit F. Rabbit D apparently died suddenly as a result of the fresh cardiac lesion. One of the cusps of the tricuspid valve was enormously thickened, forming a large tumor that could be seen through the heart wall. A stained section showed an edematous and necrotic valve filled with fibrin and bearing a large subendothelial hemorrhage at its free edge. Small clumps of streptococci were visible in the tissue. Rabbit F had an infection of the cornea of one eye which spread outward into the conjunctiva and inward into the anterior and posterior chambers of the eye, also into the choriocapillaris and retina. *Streptococcus epidemicus* was isolated repeatedly from the conjunctiva during life.

The lesions of the ears and testes appearing in such a large percentage of the rabbits injected with cultures from this epidemic seemed remarkable and more than mere coincidence. In 52 rabbits similarly injected with streptococci from other epidemics by Smith and Brown similar lesions of the ears were encountered four times and orchitis not at all. The tendency to produce these lesions in rabbits was apparently one of the "distinguishing characteristics" of strains isolated in this epidemic, and one which tended to identify the strain

from Cow 108 with those from throats. In contrast to the results reported by Rosenow (11) at various times in the elaboration of his theory of "selective localization," however, it is to be noted that erysipelas and orchitis were not reported in any of the human patients during the epidemic. The organism produced certain characteristic lesions in patients, and others equally characteristic in rabbits. It was interesting to find that the strain from Cow 108, 19 months after isolation, was still able to produce the ear lesion in Rabbit G, but orchitis was not produced.

#### *Subsequent Study of the Dairy.*

The contamination of the milk with the streptococcus from Cow 108 was something which could not be detected by current routine methods for the examination of milk. One of our objects, therefore, was to devise methods for safeguarding the production of raw milk against such accidents. With this end in view milk from the cows of Dairy H and swabs from the throats of the employees were studied for 2 or 3 months after the subsidence of the epidemic. There is nothing particularly difficult about culturing throat swabs from the employees in blood agar once a week. This, we believe, is the most important measure for the prevention of milk-borne streptococcus epidemics. Second in importance is the culturing of milk in blood agar. Obviously it is not practicable to culture the milk of individual cows each week. It was found, however, that if a mixed sample of the milk from a group of ten to fifteen cows was plated out in blood agar it was easy to detect strains of bacteria which formed the characteristic flora of milk from individual cows of the group. In the examination of milk from individual cows it was found that certain cows gave almost sterile milk while others gave milk containing large numbers of bacteria. These high counters, as we called them, seemed to harbor a characteristic flora in their milk ducts. These organisms were commonly streptococci of the alpha or gamma type in blood agar, sometimes micrococci, and more rarely bacilli or streptococci of the beta type. A small amount of the milk of Cow 108 added to a group sample was easily detected in the blood agar plate. Many times we were able to detect the withdrawal of a cow or the addition of a cow

in a certain group by a change in the group flora. If an organism appeared in a group sample which was at all suspicious, samples from the individual cows of that group were cultured. By the use of this procedure it would be easy to lower the bacterial count of a dairy milk by gradually eliminating certain cows which are high counters. In the purchase of a dairy cow there is no reason why this should not be taken into consideration.

In correlation with the regular examination of throat swabs from the milkers and group samples from the cows, the confining of a certain milker to a certain group of cows is highly desirable from a sanitary standpoint. In Dairy H this was considered impracticable but we believe that the ability to fix the responsibility for a certain grade of milk from a certain group of cows with a certain milker would have economic as well as sanitary advantages.

As a minimum requirement for dairies producing raw milk we would recommend the regular examination of throat swabs from the milkers and the use of blood agar rather than plain agar for making milk counts.

#### SUMMARY.

A streptococcus epidemic of moderate extent and severity was characterized by clinical symptoms different from the usual septic sore throat, though the organism found was culturally *Streptococcus epidemicus*.

The infection was traced to the milk from a single quarter of the udder of a cow in a dairy of 112 cows producing an otherwise excellent grade of raw milk.

A number of the milkers on the dairy farm were found infected. It was impossible to trace the infection of the cow's udder to any one of the milkers, though such an infection seems probable since the streptococcus isolated from the cow was in every respect like streptococci isolated from patients and milkers, and different from those usually found in normal cows or cows with garget.

Certain recommendations are made to safeguard producers of raw milk against the occurrence of such epidemics.

*Addendum.*

The authors are indebted to Dr. E. E. Tyzzer and Dr. Marshal Fabyan of the Department of Comparative Pathology of Harvard Medical School for the following notes regarding Cow 108 after she had passed from under our observation.

Cow 108 was kept under observation until Jan. 3, 1918, when, since lactation had ceased, she was slaughtered for beef. During this time the following bacteriological examinations of the milk were made.

Aug. 27, 1917. Milk from the left hind quarter showed the hemolytic streptococcus previously isolated.

Aug. 29. Milk from the right hind quarter, no hemolytic organism present in plates.

Aug. 30. Milk from the right fore quarter, hemolytic streptococcus not present.

Sept. 11. Right hind quarter negative.

Sept. 13. Right fore quarter negative.

Sept. 25. Both right hind and right fore quarters negative.

The infected quarter was the first to become dry and the secretion of milk had ceased in other quarters by the middle of November. The localized induration which had become apparent soon after the cow came under observation, persisted up to the time of slaughter. It was situated at some distance above the teat in the anterior portion of the left hind quarter of the udder and appeared as an ill defined mass of about the size of a hen's egg.

*Autopsy.*—The udder was first sliced in various directions by the inspector who made his examination before turning the material over for further study. The affected portion after incision showed remarkably little difference from the normal portion. The involved tissue, however, was slightly firmer and less flabby than the normal gland. The animal showed also an early tuberculosis involving the bronchial lymph nodes and a small portion of the lung.

*Microscopic Examination.*—Stained sections of various samples of the mamma showed definite inflammatory changes in the indurated part, which were absent in other portions. There appeared to be an increase in the interglandular connective tissue, although this is rather difficult to determine and may be an open question. The chief abnormality consisted of collections of lymphoid cells mingled with which were few plasma and large mononuclear cells. The larger foci were distributed in the walls of the large ducts but there were also similar foci including gland acini. The ducts showed eosin-staining secretion in which an occasional mononuclear cell was apparent, and this material differed in no way from the secretion found in the ducts of the normal quarters.

Cultures taken from the indurated portion of the gland were negative. The inoculation of rabbits with a suspension obtained by grinding this tissue in salt solution resulted negatively.

It is evident from these findings that the infection was present in the left hind quarter as late as Aug. 27, 1917. At the time of autopsy no evidence was obtained of the presence of a virulent streptococcus. The absence of polynuclear leucocytes in both the tissues and the secretion indicates that there was at this time no active process present.

#### BIBLIOGRAPHY.

1. Smith, T., and Brown, J. H., *J. Med. Research*, 1914-15, xxxi, 455.
2. Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1919, 27, 28.
3. Keegan, J. J., *J. Am. Med. Assn.*, 1919, lxxii, 1434.
4. Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253.
5. Ayers, S. H., *J. Bacteriol.*, 1916, i, 84.
6. Ayers, S. H., Johnson, W. T., and Davis, B. J., *J. Infect. Dis.*, 1918, xxiii, 290.
7. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.
8. Broadhurst, J., *J. Infect. Dis.*, 1912, x, 272.
9. Stowell, E. C., Hilliard, C. M., and Schlesinger, M. J., *Science*, 1913, xxxviii, 373; *J. Infect. Dis.*, 1913, xii, 144.
10. Brown, J. H., *J. Exp. Med.*, 1920, xxxi, 35.
11. Rosenow, E. C., *J. Infect. Dis.*, 1914, xiv, 61; 1915, xvi, 240, 367; 1916, xix, 527, 333. Rosenow, E. C., and Dunlap, S. I., *J. Infect. Dis.*, 1916, xviii, 383. Rosenow, E. C., and Moon, V. H., *J. Infect. Dis.*, 1915, xvii, 69. Rosenow, E. C., and Oftedal, S., *J. Infect. Dis.*, 1916, xviii, 477.

#### EXPLANATION OF PLATE 9.

FIG. 1. The first blood agar plate culture of milk from Cow 108 showing large numbers of colonies of *Streptococcus epidemicus*, after incubation over night. The plate was too thickly seeded for the colonies and zones to attain their full development.



FIG. 1.

(Brown and Orcutt: Dairy infection with *S. epidemicus*.)



## MYCOSIS OF THE BOVINE FETAL MEMBRANES DUE TO A MOULD OF THE GENUS MUCOR.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N.J.)

(Received for publication, December 6, 1919.)

An accurate knowledge of the number and variety of living organisms which may invade the uterochorionic space during pregnancy in bovines and multiply enough to set up localized or general disease of the chorion can only be gained by a cumulative study of the pregnant uterus before discharge of the fetus. After expulsion of the latter, adhesion of the placenta, the loss of pathological fluids held between uterine wall and chorion, the soiling of the placenta by the bedding, or its destruction by the cow, all stand in the way of an elucidation of those still unknown agencies of disease which may be of far more significance than is credited to them. The relation of *Vibrio fetus* to abortion may be considered as established. Occasionally *Bacillus pyogenes* is present in such numbers in the organs of the discharged fetus that tentatively it may be regarded as a primary agent in certain cases.<sup>1</sup>

Among a considerable number of pregnant uteruses obtained from abattoirs, the writer found one which deserves a brief description. The uterus was brought intact to the laboratory and the following facts were ascertained.

*Case 182.*—When the wall of the uterus was cut through there was found between uterine mucosa and chorion (uterochorionic space) a considerable amount of a turbid fluid full of small flakes. Over a region of the chorion about 20 cm. in diameter, corresponding with or resting on the left shoulder and thorax of the fetus, the cotyledons, five in number, are separated from the uterine wall so that the latter

<sup>1</sup> Smith, T., *J. Exp. Med.*, 1919, **xxx**, 325. Zwick and Zeller, *Arb. k. Gesundh.-samte.*, 1913, **xliii**, 1.

can be lifted away from the fetal membranes over this area. The pedicles of these cotyledons (maternal caruncles) are short, blunt projections of the uterine wall, varying in size, in color grayish with some hemorrhagic spots. The affected cotyledons are enlarged, the margins much thickened, and rolling cup-shaped over into the central necrotic portion. The chorion between these detached cotyledons is beset with yellowish gray, slightly elevated plaques, 2 to 4 mm. in diameter and a fraction of a mm. thick, producing a coarse goose-skin appearance. The subchorionic edematous tissue appears as a glassy, gelatinous layer 1 to 2 cm. thick. In one horn the chorion, over an area 6 cm. square, is beset with grayish to yellowish plaques containing minute hemorrhages. The fluid in the allantois at both horns is clear. The amniotic fluid contains a fairly abundant amount of fecal matter. The cervix of the uterus is normal and the external os is tightly closed and provided with the normal mucous plug.

The fetus is 25 inches long and normal, with the following exceptions. There is edema of the sheath of the umbilical vein within the abdomen, also slight edema of the capsule of the kidneys, and of the interlobular tissue of the lungs. The rumen contains considerable yellowish (fecal) matter, but the fourth stomach contents are still clear and colorless.

Although the gross appearances did not correspond closely to those found in the presence of *Bacillus abortus*, the case was considered one of infectious abortion due to this bacterium, until examination of the scrapings of the cotyledons showed a branched mycelium and no bacteria of any description. Cultures on slanted agar, sealed with sealing-wax and unsealed, of material from the surface of the chorion and from the amniotic fluid developed a mould. Cultures from the lungs and meconium of the fetus also contained only a mould, while those from the fourth stomach, spleen, liver, and one kidney remained sterile. Subsequent study of the moulds from placental and fetal cultures showed them to be identical and belonging to the genus *Mucor*.

The absence of *Bacillus abortus* from the cultures was confirmed by the results of inoculation of guinea pigs with the fluid exudate in the uterochorionic space, with amniotic fluid, and with contents of the fourth stomach of the fetus. The guinea pigs killed after 26 days

were normal and cultures remained sterile. Films from the chorion and the fluid exudate showed no bacteria of any kind. In the necrotic masses scraped from the cotyledons, a branched, non-septate mycelium was detected.

Tissues fixed in Zenker's fluid from various diseased and normal regions of the placenta were studied in sections in order to determine the relation of the mould to the tissues and the character of the tissue reaction. Although a variety of dyes was used, the mycelium stained very poorly and only careful focusing with a partly closed condenser made it possible to identify the filaments and trace them as far as the section permitted.

The remnants of the caruncles projecting from the uterine wall were densely infiltrated and covered with masses of polynuclear leucocytes. Among these masses the branched mycelium could be traced, penetrating them in various directions. The corresponding cotyledons with the sloughed off portions of the caruncles still embedded in them presented a variety of changes, consisting chiefly of necrosis of most of the tissue and masses of polynuclear leucocytes. Traces of the original tissue appeared in islands of villi, densely filled with blood corpuscles. Branched mycelium could be seen penetrating the necrotic and purulent foci. It varied somewhat in diameter and was more or less angular and dilated in places. Most of the filaments appeared empty, at least the staining did not reveal any contents. No fruiting bodies, either in the form of sporangia or chlamydospores, were found.

As stated above, the intercotyledonous areas of the affected region of the chorion were beset with hemispherical elevations about 2 to 4 mm. in diameter, which gave the membrane a coarse goose-skin appearance. The sections showed that over the elevations it was deprived of its epithelium and the subjacent, bared zone filled with dense masses of nuclear debris which projected somewhat and gave the surface its nodular outline. This lesion is similar to that found in the bared chorion when *Bacillus abortus* is the agent, as well as in cases associated with *Vibrio fetus*. It would seem as if any injury to or destruction of the epithelium leads to a movement of leucocytes towards the surface, under which they gather in dense groups and disintegrate.

The invasion of the fetal membranes by a mould presupposes some primary focus in the body of the dam, whence spores might enter the circulation and break through into the fetal cotyledons. Unfortunately nothing is known of the dam, since the cow was slaughtered in the routine work of the abattoir and only the pregnant uterus reserved. If the above view of the source of the infection is correct it would imply the existence of moulds in the lungs of the cow where the oxygen requirements are sufficient for the production of sporangia and spores.

The literature on pathogenic mucors is very meager as contrasted with that on *Aspergillus*. Lichtheim<sup>2</sup> isolated from moistened bread two species pathogenic for rabbits after intravenous injection of fairly large doses of spores, *Mucor rhizopodiformis* and *Mucor corymbifer*. The chief loci of germination and growth were the kidneys, the lymphoid tissue of the intestines, and the mesenteric nodes. Paltauf<sup>3</sup> soon afterwards found a mucor in focal lesions of the cerebrum, cerebellum, lungs, pharynx, and ileum of a man 52 years old. The mucor was not cultured. The dimensions given indicate that he had a much smaller type under observation than the types mentioned above. Lindt<sup>4</sup> isolated two additional species from moistened, incubated wheat bread which were pathogenic for rabbits after intravenous injection of spores. These were named *Mucor pusillus* and *Mucor ramosus*, both distinguishable from one another and from the species cultured by Lichtheim on account of the shape and dimensions of the spores. Hückel<sup>5</sup> isolated a mucor from a cerumen plug taken from the ear of a patient 28 years old. This he identified with *Mucor corymbifer*.

#### *Biological and Pathogenic Characters.*

The mould was readily cultivated at incubator temperature on ordinary nutrient agar, although an addition of 1 per cent dextrose increased the vigor of the growth. At 70°F. the growth was much

<sup>2</sup> Lichtheim, L., *Z. klin. Med.*, 1884, vii, 140.

<sup>3</sup> Paltauf, A., *Virchows Arch. path. Anat.*, 1885, cii, 543.

<sup>4</sup> Lindt, W., *Arch. exp. Path. u. Pharmakol.*, 1886, xxi, 269.

<sup>5</sup> Hückel, A., *Reitr. path. Anat. u. Physiol.*, 1886, i, 117.

slower. It presented the following characters. A dense, branching, silken, whitish mycelium spreads over the surface and, in tubes, penetrates vertically for a distance of 5 to 10 mm. into the depths of the agar. The mycelium varies from 4 to  $10\mu$  in diameter. It contains a slightly refractile, coarsely or finely granular cytoplasm. The branching of the mycelium was at no time observed to follow any definite scheme. It evidently depended on the condition of the substrate.

In addition to this adherent layer of interlacing filaments an abundant aerial woolly mass of filaments appeared within 48 hours. These were found empty and often collapsed later on, probably as a result of drying. Rhizoids, or radiations of short filaments from centers of growth on the agar surface, were found, from which initiating hyphæ arise to bear at the free end the sporangia. These sporangiophores and the rhizoids can be distinguished from the vegetative mycelium by their brownish tint under a low power. The rhizoid gives origin to a short trunk which soon divides irregularly into a small number of sporangiophores.

The sporangia, barely visible to the unaided eye, become a grayish brown color as they ripen and appear almost black under a low power in transmitted light. They are subspherical, measuring transversely about  $80\mu$  and vertically about  $64\mu$ , although smaller heads occur. The columella seen after discharge of the spores projects as a spherical body from the funnel-shaped, expanding end of the supporting hypha. The slightly brownish spores are spherical and measure about  $4.5\mu$ , although some as small as  $4\mu$  and as large as  $5\mu$  may be seen.

The mycelium on the agar surface may form in places two transverse septa close together. The intervening walls of the filaments bulge and the segment thus isolated and containing a granular cytoplasm forms a rather thick wall showing later on a double contour. The filaments on both sides of this new body shrivel. In rare instances a series of such segregated and encysted masses of cytoplasm may form. These bodies, usually denominated chlamydospores (*Gemmen, Dauerzellen*), were quite numerous in all cultures. No zygospores were seen at any time. An examination of the literature indicates that Lichtheim's *Mucor rhizopodiformis*<sup>2</sup> comes nearest to

the species under investigation, although there are certain differences such as a colorless, slightly larger spore (5 to  $6\mu$ ) in Lichtheim's culture.

No special studies were made upon the behavior of this species under different environmental conditions. This much was frequently observed, however, that the mould is very capricious in the production of sporangia. Apparently the same conditions at one time lead to a rapid, copious formation of sporangia with ripening of spores in 2 to 3 days, in another to none. The obvious interpretation is that slight unrecognized differences in the environment exercise a decisive influence. Evidently oxygen is an important factor, for the sealing of tubes, which reduces the oxygen tension through absorption of the oxygen by the culture medium and by the mould during the early stages of growth, interferes with spore production.

To determine whether spores of the mould would germinate and develop a mycelium in the organs of rabbits, the growth from a dextrose agar culture was thoroughly stirred about in sterile bouillon and filtered through five layers of sterile fine bandage material to remove fragments of mycelium and clumps of spores. The resulting feebly clouded, lightly brownish fluid was found with only isolated spores. Doses of 1.5 and 0.5 cc. were injected into an ear vein of two rabbits weighing 2,218 and 2,404 gm. respectively. The rabbit receiving the smaller dose was in appearance normal until the 8th day, when it was chloroformed. The weight had dropped from 2,404 to 2,286 gm. The spleen was a trifle large and congested. It was beset with numerous subcapsular, 0.5 mm., isolated or agglomerated, whitish, slightly projecting nodules. Both kidneys show the same kind of nodules, about 1 to 5 mm. apart. Some of these correspond to opaque whitish streaks passing through the medulla and continuing in slenderer radial lines to the pelvis. In the latter no changes are evident. Scattering nodules occur in the liver and one in a Peyer's patch. The appendix is free. Several mesenteric nodes contain large necrotic foci. In one kidney nodule, crushed between slides, branching mycelium could be traced for some distance. Bits of spleen, liver, and kidney tissue placed in agar slants developed a rich mycelium within 24 hours.

The second rabbit died unexpectedly on the 7th day. No disturbance of health had been noticed but the weight at death was only 1,740 gm. Lesions much smaller than in the first rabbit were found in spleen and kidneys. The spleen was small and pale. The cause of death was probably some intercurrent influenza, since the lungs were generally congested and edematous and the nostrils ringed with dry crusts. There was, however, no similar disease among the older stock animals and it may be that the resistance had been lowered by the mould.

Sections of tissue of the first rabbit fixed in Zenker's fluid presented a number of interesting details which can only be briefly enumerated. The lesions of the kidneys in the cortex were made up of dense collections of polynuclear leucocytes within areas in which the interstitial cells had also proliferated. The affected area included one or several glomeruli and the tubules immediately surrounding them. The origin of the lesion, whether in a glomerulus or the tubules, could no longer be determined, as the original structures were barely identifiable. The focus thus begun could be traced downward, the associated tubules being filled with masses of partly disintegrated cells, chiefly polynuclears. These plugs extend to the tip of the papilla. Here the pelvis contains a mass of polynuclears completely surrounding the papilla. Branched mycelium could be seen in all the involved tubules. It was most abundant in the cellular masses of the pelvis. The changes in the spleen were similar to those in the cortex of the kidney. In the mesenteric nodes the foci were so numerous as to coalesce and involve more than half the node. Mycelium was specially abundant. In the liver two kinds of lesions were present, collections of polynuclear leucocytes taking the place of destroyed liver tissue and much smaller foci composed of multinucleated cells, some closely resembling those of the tubercle. No formed elements could be made out within these giant cells, but it is probable that they contained mould spores. Microscopic foci were not found in the lungs although all the injected spores must have passed through these organs.

These two inoculations serve to place this mucor among Lichtenheim's so called pathogenic species. There are certain minor differences between his results and the present, which might disappear in

larger series. The slightly lower pathogenic effect when compared with Lichtheim's tests may be due to the fact that the inoculations were made after the mould had been kept 23 months on culture media.

The source of the infection in the cow can only be conjectured. The two widely distributed mucors, not pathogenic for rabbits, *Mucor mucedo* and *Mucor racemosus*, occur frequently in dairy products,<sup>6</sup> and it is probable that the species under consideration may be found there and thus represent a possible source of the infection.

#### SUMMARY.

A mucor, closely resembling Lichtheim's *Mucor rhizopodiformis*, was isolated from the diseased chorion of a cow and from the lungs and digestive tract of the fetus. No other microorganisms were detected. The mucor was demonstrated in teased preparations from the fresh cotyledons as well as in sections of tissues suitably hardened. It produced focal lesions in rabbits following the intravenous injection of spores. The condition of the amniotic fluid and the contents of the rumen of the fetus justify the inference that premature expulsion was impending.

<sup>6</sup> Weigmann, H., in Sommerfeld, P., *Handbuch der Milchkunde*, Wiesbaden, 1909, 328.

## SOURCE AND SIGNIFICANCE OF STREPTOCOCCI IN MARKET MILK.

BY F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, January 28, 1920.)

Considerable discussion has arisen as to the source and significance of streptococci in milk. Most of the observations were made before the use of the blood agar plate method or before the use of more accurate determinations of acid production became a common practice. New interest in the problem has been aroused since the outbreaks of septic sore throat attributed to contamination of the milk supply with streptococci pathogenic for man. A more thorough knowledge of milk streptococci, particularly those producing hemolysis in blood agar plates, is necessary. The source of such organisms is of particular interest.

Rogers and Dahlberg<sup>1</sup> undertook the study of milk streptococci in detail. They examined mixed milk, udder milk, feces, and saliva of cows. Blood agar plates were not used. The bouillon employed was prepared from beef extract to which dextrose, lactose, saccharose, mannite, raffinose, starch, and glycerol were added to make 1 per cent solutions. In all a large number of cultures were studied. The fecal streptococci were characterized by their ability to attack the sugars, including raffinose, and their inability to utilize alcohols. The salivary strains fermented dextrose, lactose, saccharose, mannite, and frequently raffinose. The amount of acid produced was lower than that obtained from the fecal streptococci. They divided the udder streptococci into two groups on the basis of the liquefaction of gelatin. One group, which they considered indistinguishable from *Streptococcus pyogenes*, fermented dextrose, lactose, and saccharose with an occasional culture breaking down mannite, starch, or inulin. In addition to liquefying gelatin, the other group attacked dextrose, lactose, saccharose, mannite, and generally fermented glycerol. From these studies the conclusion was reached that the streptococci found in market milk were of udder origin.

---

<sup>1</sup> Rogers, L. A., and Dahlberg, A. O., *J. Agric. Research*, 1913-14, i, 491.

Davis<sup>3</sup> in examining the market milk from nine dairies found hemolytic streptococci in eight samples; eight of the samples had been pasteurized. One of the pasteurized samples did not contain hemolytic streptococci. In the other seven they made up from 5 to 45 per cent of the flora. In one sample of certified (raw) milk 40 per cent of the bacteria were hemolytic streptococci. The data concerning fermentation are given for ten strains. They are compared with three strains of human origin and one culture isolated from a case of bovine mastitis. All the milk streptococci fermented dextrose, lactose, maltose, and salicin. Seven acidulated saccharose and one mannite. The titratable acidity in dextrose bouillon varied from 5.05 to 6.55 per cent. Two of the human cultures produced somewhat less acid than the milk strains (4.55 and 4.70 per cent), but the other produced more acid (6.25 per cent). The mastitis strain could not be differentiated by its cultural characters from the milk streptococci. However, it possessed considerable pathogenicity for rabbits. Davis points out that of the 85 strains studied none had properties which would justify his considering them of human origin. He concludes by stating that the milk strains are different from certain strains of hemolytic streptococci found at times in the diseased udders of cows, since the latter are virulent for rabbits and are from human sources.

The writer<sup>3,4</sup> has shown that apparently normal cows may harbor in the udder, streptococci which are identical in all cultural characters and agglutination affinities with those causing mastitis. It was pointed out that in a herd in which a large number of cows suffered from mastitis many cows carried streptococci in the udder. From this observation it was assumed that many such streptococci would gain access to the milk supply.

To establish the possible types of streptococci which may appear in market milk, examinations of the vaginal discharges, saliva, feces, and skin of cows in a large herd were undertaken. The milk from this herd is of high quality and is sold in one large city as certified milk. In another city it is marketed as Grade A raw milk which is certified by a board of physicians. The usual precautions taken in the production of certified milk are maintained.

#### *The Possible Sources of Streptococci.*

In a previous paper<sup>4</sup> the vaginal streptococci were considered in detail. At that time it was noted that hemolytic streptococci were not found on the normal vaginal mucosa. A considerable number

<sup>3</sup> Davis, D. J., *J. Infect. Dis.*, 1916, xix, 236.

<sup>4</sup> Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253.

<sup>4</sup> Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 735.

of the non-hemolytic variety were isolated and studied. They fell into two principal groups, those attacking dextrose, lactose, saccharose, maltose, and mannite, and those fermenting the first four sugars but not fermenting mannite. Salicin fermentation predominated in each group.

Swabs were employed in isolating streptococci from the mouth and skin. The swabs were introduced into the mouth and rubbed over the tongue and mucous membranes. Within an hour they were agitated in 10 cc. of sterile 0.9 per cent sodium chloride solution. From this suspension three platinum loopfuls were inoculated into 12 cc. of melted 2 per cent agar prepared from veal infusion which had been cooled to 45°C. The mixture was plated with 1 cc. of defibrinated horse blood. The plate cultures were examined after incubation for 24 hours at 38°C.

For the skin examinations the lumbar region was chosen. Since the cows were confined in stanchions the possibility of contaminating this area with either feces or saliva was not considered great.

Feces were obtained as early after defecation as possible and suspended in the usual amount of salt solution and plated at once.

The cultures were inoculated into tubes containing 13 cc. of fermented veal infusion broth (+ 0.6 to + 0.8 phenolphthalein) to which the test substances were added to make a 1 per cent solution. Titrations were made after 5 days incubation at 38°C.

In all, the saliva, skin, and feces of 45 cows were examined. Hemolytic streptococci have not been isolated from these regions. The saliva contains many streptococci. In all, thirty-seven strains have been isolated and studied. They comprise, however, an exceedingly heterogeneous group. Mannite fermentation is frequent and the proportion of raffinose and inulin fermenters is high. As a rule, low acid production in dextrose is characteristic. A tabulation of their characters has been omitted as it will be shown later that such streptococci have not been found in the milk.

From the skin thirteen cultures of streptococci have been isolated. Their cultural characters, given in Table I, are more uniform than those from the mouth. From Table I it will be noted that the characteristic streptococcus from the skin is a type which produces short chains in bouillon, acidulates milk, and ferments dextrose, lactose,

saccharose, maltose, raffinose, mannite, and salicin. Mannite is uniformly attacked to a lesser degree than the other substances. Inulin is not fermented. Gelatin is not liquefied.

TABLE I.  
*The Non-Hemolytic Streptococci from the Skin.*

Culture No.	Grouping.*	Growth in bouillon.	Litmus milk.	Production of acid in.							
				Dextrose. per cent	Lactose. per cent	Saccharose. per cent	Maltose. per cent	Raffinose. per cent	Inulin. per cent	Mannite. per cent	Salicin. per cent
Sk 2	Pairs; chains of 4.	Turbid.	Coagulated on boiling.	2.7†	2.4	2.8	2.7	2.6	0.2	0.9	2.8
" 11	Pairs and S. C.	"	" "	3.3	2.9	2.9	3.2	2.7	0.2	1.2	3.2
" 12	" "	"	" "	3.2	2.6	3.5	3.2	2.9	0.0	2.0	3.0
" 20	" "	"	Acid.	3.1	2.6	3.2	2.7	2.9	0.2	2.5	3.2
" 23	" "	"	Coagulated on boiling.	3.2	2.9	3.3	3.0	2.8	0.1	1.8	3.1
" 24	" "	"	" "	2.9	2.5	2.9	2.8	2.3	0.1	2.5	2.7
" 26	" "	"	" "	2.4	2.4	2.2	2.2	2.4	0.0	1.0	2.4
" 27	" "	"	" "	4.3	3.3	3.3	3.6	3.1	0.0	2.5	4.0
" 29	" "	"	" "	4.2	3.6	4.1	4.3	3.5	0.0	1.2	3.3
" 30	" "	"	" "	2.6	2.1	2.4	2.6	2.5	0.0	2.9	2.4
" 32	" "	"	" "	2.7	2.2	2.6	2.4	3.0	0.0	1.3	2.4
" 34	" "	"	" "	3.6	2.9	3.3	3.1	2.8	0.0	1.4	2.7
" 35	" "	"	" "	3.0	2.7	2.6	2.9	2.7	0.0	1.0	2.7

\* The length of chains has been indicated as follows: S. C., 4 to 8 cocci; M. C., chains of 8 to 16; L. C., chains of more than 20.

† Only net acid production has been recorded in the tables. The addition of 0.7 to the figures in the various columns will give a close approximation of the total acidity.

Twenty-one strains of streptococci have been isolated from the feces. Their cultural characters are shown in Table II. The bulk of fecal streptococci fall into one large group. This group is characterized by the formation of large amounts of acid in dextrose, lactose, saccharose, maltose, raffinose, inulin, and salicin. Litmus milk is firmly coagulated and litmus is usually reduced. Gelatin is not liquefied. Two cultures, Nos. F 11 and F 20, belong to the skin group.

Since the vaginal, skin, and fecal streptococci possess specific characters rendering their classification definite, each type should be readily recognized if found in milk.

TABLE II.  
*The Non-Hemolytic Fecal Streptococci.*

Culture No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in							
				Dextrose. per cent	Lactose. per cent	Saccharose. per cent	Maltose. per cent	Raffinose. per cent	Inulin. per cent	Mannite per cent	Salicin. per cent
F 4	Pairs and S. C.	Turbid.	Firmly coagulated.	7.3	5.1	6.5	6.3	6.3	7.3	0.0	5.5
" 6	" "	"	" "	8.3	4.9	7.1	7.6	7.0	6.5	0.0	5.0
" 18	" "	"	" "	7.2	5.4	5.6	7.0	5.8	7.0	0.0	5.0
" 21	" "	"	" "	6.4	4.6	5.7	5.7	5.7	6.6	0.0	5.5
" 28	" "	"	" "	6.4	5.0	5.5	5.9	5.7	6.6	0.1	5.0
" 29	" "	"	" "	5.3	4.4	5.2	4.9	5.1	6.0	0.0	4.5
" 30	" "	"	" "	5.7	4.2	5.3	5.7	5.5	5.7	0.0	5.3
" 31	" "	"	" "	6.5	6.4	5.8	5.8	5.1	6.4	0.0	6.0
" 32	" "	"	" "	6.2	4.0	6.0	5.6	6.0	6.7	0.0	5.2
" 33	" "	"	" "	7.2	4.5	5.9	5.8	5.7	6.4	0.0	5.4
" 34	" "	"	" "	5.9	4.6	5.4	5.8	5.8	6.0	0.0	5.4
" 35	" "	"	" "	5.9	4.3	5.8	5.9	6.5	6.5	0.0	5.6
" 36	" "	"	" "	6.4	4.7	5.7	6.1	4.9	6.3	0.0	5.1
" 37	" "	"	" "	6.0	4.2	6.0	5.2	5.4	6.0	0.0	4.6
" 39	" "	"	" "	6.4	4.5	5.3	4.5	6.5	6.0	0.0	5.3
" 40	" "	"	" "	6.4	4.0	5.5	5.3	6.1	6.3	0.0	5.1
" 2	" "	"	" "	7.4	5.8	5.9	6.2	3.3	0.0	0.0	5.8
" 11	" "	"	Coagulated on boiling.	3.2	2.4	3.1	3.1	2.9	0.1	1.8	3.0
" 20	" "	"	Acid.	3.9	3.0	2.7	3.1	3.0	0.1	1.4	3.5
" 16	M. C.	"	Unchanged.	4.1	0.0	4.1	4.3	3.2	2.8	3.4	4.0
" 46	Pairs and S. C.	"	Acid.	2.1	2.0	2.1	2.0	2.0	0.0	0.2	2.0

#### *Examination of Market Milk for Streptococci.*

Pint bottles of milk from the herd supply were chosen at random at various times. 1 cc. of milk was diluted in 9 cc. of sterile salt solution. After shaking vigorously, 0.5 cc. of the mixture was plated with 12 cc. of melted agar and 1 cc. of defibrinated horse blood. After incu-

bation for 24 hours at 38°C. the plates were examined. In all, twenty-six samples have been plated. The average for all the samples has been 2,850 organisms per cubic centimeter, of which 15.5 per cent have been streptococci. The proportion of streptococci varied over a wide latitude; the greatest number recorded was 45 per cent of the

TABLE III.  
*The Non-Hemolytic Streptococci from Milk.*

Culture No.	Grouping.	Growth in bouillon.	Litmus milk.	Production of acid in.								Results of agglutination with mastitis streptococcus, serum dilution 1: 1,000.	
				Dextrose. per cent	Lactose. per cent	Saccharose. per cent	Maltose. per cent	Raffinose. per cent	Inulin. per cent	Mannite. per cent	Salicin. per cent		
BM 8	L. C.	Clear.	Firmly coagulated.	3.4	2.8	2.8	3.0	0	0	0.1	2.3	+	
" 13	"	"	" "	4.5	3.5	3.2	3.8	0	0.1	0.3	1	-	
" 17	"	"	" "	4.5	3.4	3.3	3.0	0	0.1	0.3	2	(1: 500 = ++)	
" 27	M. C.	Turbid.	" "	4.3	3.6	3.3	3.4	0	0.1	0.3	2	+++	
" 32	L. C.	"	" "	4.7	3.9	3.4	3.8	0	0.0	0.0	2.9	+++	
" 39	"	"	" "	4.4	3.8	3.8	4.0	0	0.1	0.3	2	-	
" 49	M. C.	"	" "	3.8	3.8	3.1	3.2	0.1	0.1	0.2	9	(1: 500 = +)	
" 53	L. C.	"	" "	4.4	3.9	3.9	4.0	0	0.0	0.0	3.8	+++	
" 61	"	"	" "	4.5	3.6	3.5	4.1	0.1	0.1	0.1	3.8	+	
" 69	S. C.	"	" "	4.6	3.7	4.4	4.2	0	0.1	0.0	3.7	-	
" 72	M. C.	Clear.	" "	4.5	4.1	4.1	3.8	0	0.1	0.0	2.8	+	
" 74	L. C.	"	" "	4.6	3.5	3.2	4.0	0	0.0	0.0	3.3	++	
" 78	M. C.	Turbid.	" "	4.6	4.0	3.3	3.8	0	0.0	0.0	3.5	++	
" 89	L. C.	"	" "	4.3	3.5	3.5	4.0	0	0.0	0.0	2.9	++	
" 92	M. C.	"	" "	4.3	3.5	3.3	3.5	0	0.0	0.0	3.3	+++	
" 23	S. C.	"	Coagulated on boiling.	3.0	2.9	3.1	2.7	2.8	0	0.1	0.3	1	-

\* +++ indicates complete agglutination; ++, marked clumping without entire clearing of the fluid; +, moderate agglutination. A negative reaction is noted as -.

total number of organisms. Of the 72 streptococci isolated, 56 are of the hemolytic type. The others are non-hemolytic. These figures indicate a greater proportion of hemolytic streptococci, although this is not necessarily the case. The hemolytic colonies are more

easily differentiated from the others and many of the deeper non-hemolytic colonies may have been overlooked. In Tables III and IV the cultural characters of the milk streptococci are given.

It will be observed that the non-hemolytic streptococci with one exception fall into the mastitis group. They all produce considerable acid in dextrose, lactose, saccharose, maltose, and salicin. Milk is firmly coagulated but litmus is not reduced. In addition, all but one agglutinate with their specific group serum. This serum was obtained by the immunization of a cow with a strain of non-hemolytic mastitis streptococcus. Strain BM 23 agrees in neither its cultural nor immunological characters with the other organisms; it is probably from the skin, since it ferments dextrose, lactose, saccharose, maltose, raffinose, mannite, and salicin.

Of 56 strains of hemolytic streptococci, 43 agree in their cultural characters and agglutination affinities with the hemolytic types most frequently associated with bovine mastitis. Like the non-hemolytic group, they produce considerable acid in dextrose, lactose, saccharose, and maltose. Salicin may or may not be fermented. Milk is firmly coagulated without the reduction of litmus. These high acid-producing strains (both hemolytic and non-hemolytic) occurred in the milk in large numbers. They make up the bulk of the streptococcal flora.

The cultural characters of the other thirteen strains differ from those associated with mastitis. They ferment dextrose, lactose, saccharose, and maltose, but none has attacked inulin, raffinose, mannite, or salicin. One culture fails to attack saccharose. Milk is coagulated but not firmly. The curd is flocculent. Litmus is often partially reduced. The amount of acid produced in dextrose is considerably lower than that recorded for the mastitis type. In addition, the hydrogen ion concentration is higher, pH 5.0 to 5.3. Gelatin and coagulated serum are not liquefied. An additional point of differentiation between members of this group and those of the mastitis group is the difficulty with which they are suspended in 1 per cent sodium chloride solution. The mastitis strains all readily enter into suspension. The others sediment spontaneously within 3 or 4 hours. When freshly isolated many of these strains grow with difficulty in fermented or plain bouillon, even when carbohydrate is added; the addition of 5 to 10 per cent of sterile horse serum to the media insures

## STREPTOCOCCI IN MARKET MILK

TABLE IV.  
*The Hemolytic Streptococci from Milk.*

Culture No.	Growth Grouping.	Littmus milk.	Production of acid in.	Results of agglutination test with mastitis streptococci, serum dilution 1:200.								Hydrogen ion concentra- tion in dextrose, fermented broth.	$\text{pH}$	
				Dextros.	Lactose.	Saccharose.	Maltose.	Raffinose.	Mellitose.	Galactose.	Fructose.	Salicin.		
BM 1	M. C.	Turbid.	Firmly coagulated.	4.7	3.8	3.9	4.3	0.0	0.0	0.0	0.1	0.1	++	4.4
" 5	"	"	"	4.7	3.8	4.0	4.3	0.1	0.1	0.1	0.1	0.1	++	4.5
" 6	"	"	"	4.5	3.9	3.8	4.0	0.1	0.1	0.0	0.0	0.0	++	4.7
" 9	S. C.	"	"	4.2	3.8	3.3	3.5	0.1	0.0	0.0	0.0	0.0	++	4.7
" 10	M. C.	"	"	3.9	3.6	3.9	4.0	0	0	0.0	0.0	0.0	++	4.7
" 11	"	"	"	4.6	3.9	3.5	4.0	0.1	0.1	0.0	0.0	0.0	++	4.5
" 14	"	"	"	5.0	4.1	4.0	4.1	0.1	0.1	0.0	0.0	0.0	++	4.5
" 15	L. C.	"	"	4.7	4.0	4.1	3.6	0.0	0.1	0.0	0.0	0.0	++	4.5
" 16	"	"	"	4.4	3.8	4.0	4.2	0.1	0.1	0.0	0.0	0.0	++	4.5
" 18	"	"	"	4.8	3.7	3.9	4.2	0.1	0.0	0.0	0.1	0.1	++	4.5
" 19	"	"	"	4.0	3.7	3.8	3.9	0	0.2	0.1	0.1	0.1	++	4.5
" 20	M. C.	"	"	4.6	3.7	3.9	4.0	0.0	0.1	0.0	0.1	0.1	++	4.6
" 21	L. C.	"	"	4.9	3.8	3.9	4.3	0.0	0.0	0.1	0.1	0.1	++	4.6
" 25	"	Clear.	"	4.1	3.2	3.5	3.5	0.0	0.1	0.0	0.0	0.0	++	4.6
" 33	M. C.	Turbid.	"	4.0	3.8	3.6	3.6	0.0	0.1	0.0	0.0	0.0	++	4.6
" 35	"	"	"	4.9	4.0	3.9	4.2	0.0	0.0	0.0	0.1	0.1	++	4.6
" 37	L. C.	"	"	5.4	3.8	3.8	4.6	0.0	0.0	0.0	0.0	0.0	++	4.6
" 40	S. C.	"	"	4.6	3.9	4.0	4.3	0.1	0.0	0.1	0.0	0.0	++	4.6
" 42	M. C.	"	"	4.5	4.0	3.9	4.0	0.0	0.0	0.0	0.0	0.0	++	4.6
" 43	"	"	"	5.3	4.4	4.4	4.8	0.0	0.0	0.1	0.0	0.0	++	4.6
" 45	"	"	"	4.9	4.0	4.0	4.0	0.0	0.0	0.0	0.0	0.0	++	4.6
" 46	S. C.	"	"	4.8	4.0	4.0	4.1	0.0	0.0	0.0	0.0	0.0	++	4.6
" 47	L. C.	"	"	4.5	3.9	3.9	4.3	0.0	0.0	0.0	0.0	0.0	++	4.6



moderate growth. Growth, however, takes place slowly. In a number of instances several attempts were made before fermentation was obtained in saccharose. The colonies in blood agar plates are smaller than those produced by the members of the larger group. The surface colonies rarely reach 1 mm. in diameter. They are sharply raised and surrounded by a clear zone of hemolysis. The deep colonies are exceedingly small, biconvex or ovoid in shape, and produce clear hemolytic areas 1.5 to 4 mm. in diameter after 24 hours incubation. After 48 hours the diameter of the hemolytic area is frequently doubled or trebled.

Considerable differences in the morphology have been observed. Chains of 6 to 15 elements are usually observed in liquid media. The individual elements are usually round, slightly elongated, and even rod-shaped. In the water of condensation of blood agar cultures the organisms appear as diplococci and short rods. Clubbed ends are frequently observed. All forms retain the stain by Gram's method. The morphology of strains grown on artificial media for some time becomes more fixed, since the organisms grow in bouillon as typical short chained streptococci.

Five of the cultures from this group chosen at random were tested for pathogenicity in rabbits. 1 cc. of a 24 hour serum bouillon culture was injected into the ear veins. The animals were not appreciably affected. Their temperatures ranged well within the normal limits. Two of the rabbits were killed 15 days after injection. Necropsy failed to reveal localization either in the heart valves, the joints, or the viscera. Eight other cultures in 0.1 cc. doses were injected into the peritoneal cavities of white mice. The mice remained well.

Members of this group appeared in half the samples of milk examined, but in very small numbers; usually only one, two, or three colonies developed in plate cultures.

The low acid-producing strains in market milk had not been found in any of the regions examined. In previous studies such organisms had not been noted in plates made directly from udder milk. It was determined to trace their source back from the bottled milk. On this particular dairy farm it is customary to collect the milk from individual cows into 40 quart cans. These cans are removed from the barns, and sent to the creamery as soon as they are filled. The con-

tents of the cans, on the average, are made up of the mixed milk of five or six cows. Samples were obtained from the various cans from each barn. Characteristic colonies of the low acid-producing streptococci were observed in certain samples of can milk. This observation pointed to one of three sources of entrance: udder infection, exfoliations from the skin of the udder, or contamination by the milker during milking. Accidental contamination from exfoliations from the skin or from the milker did not seem probable, especially as covered milk pails are used. The skin of the udder of 50 cows was examined with negative results. It was determined to examine the udder milk from cows whose mixed milk was known to contain streptococci of the type sought. The search proved more difficult than at first supposed. Many udders were found infected with the typical bovine types, but comparatively few harbored the low acid producers. Of 50 cows in one barn the milk from two revealed organisms of this type. Usually, however, one finds at least one in 50 harboring the low acid-producing streptococci. The number of colonies which develop from 1 cc. of milk is usually small (40 to 80). In one instance, however, plates from the milk revealed 900 colonies per cubic centimeter. Undoubtedly more udders are invaded than indicated, since these streptococci are exceedingly delicate and are crowded out in the plates by the ordinary udder streptococci and other organisms. Higher dilutions in milk with high counts entirely eliminate them from the plate cultures since they occur only in small numbers.

Another streptococcus similar in many respects to the low acid-producing strains was found in milk drawn directly from the udder. It, however, ferments mannite and salicin in addition to dextrose, lactose, saccharose, and maltose. Such strains are also low acid producers and grow poorly in plain or fermented bouillon. The colonies and hemolytic characters in blood agar plates are indistinguishable from those noted for the low acid-producing streptococci.

#### DISCUSSION.

The predominating types of streptococci in the market milk are those of mastitis. It is true that fecal and skin streptococci may gain access to the milk, but they are practically absent in fresh milk

if it is kept under proper conditions. It is conceded, however, that certain of these streptococci may play an important part in the souring of milk; as for instance, the high acid-producing fecal strains.

Heinemann<sup>5</sup> in considering the significance of streptococci in milk came to the conclusion that *Streptococcus lacticus*, supposedly the most common type of milk streptococci, agrees in its morphological and cultural characters with pathogenic, fecal, and sewage streptococci. Miller<sup>6</sup> reached the same conclusion. He asserted that *Streptococcus pyogenes* and *Streptococcus lacticus* are indistinguishable by present methods of study. Rogers and Dahlberg reached the same conclusion in regard to certain udder streptococci.

The most frequent types of streptococci which I have observed are identical with those causing mastitis. It has been shown repeatedly that such streptococci are found in the udder before clinical manifestations of the disease appear and may persist for long periods after symptoms have subsided. That such streptococci have been isolated from market milk is shown by Davis' protocols. The only point of difference between his milk streptococci and a strain from a case of mastitis was in the virulence of the latter for rabbits. The writer has never isolated strains of mastitis streptococci of purely bovine origin which possessed high pathogenic properties for rabbits. The question is raised whether or not many belonging to the so called *Streptococcus lacticus* group may not be identical with mastitis streptococci.

The experiments of Mathers<sup>7</sup> have a distinct bearing on this point. He injected human, mastitis, and milk streptococci into the udders of cows. The human and mastitis strains produced a severe mastitis which became chronic. The non-hemolytic *Streptococcus lacticus* gave rise to an acute mastitis which displayed all the symptoms observed in spontaneous mammitis (swelling, heat, pain, and purulent milk). The injection of a culture of a hemolytic streptococcus from milk gave much the same result. The inflammation resulting from these injections disappeared within 2 weeks. The fact remains, however, that both supposedly non-pathogenic cultures produced disease. It is admitted that the inflammation is of short duration, but such is not infrequent in certain cases of spontaneous infection.

---

<sup>5</sup> Heinemann, P. G., *J. Infect. Dis.*, 1906, iii, 173.

<sup>6</sup> Miller, W. W., *Bull. Hyg. Lab., U. S. P. H.*, No. 41, 1908, 479.

<sup>7</sup> Mathers, G., *J. Infect. Dis.*, 1916, xix, 222.

It is difficult to harmonize the results obtained by Savage<sup>8</sup> in England with those obtained by me. The method of handling and caring for cows, the precautions taken to avoid contamination of the milk, etc., make such comparisons impossible. The types of streptococci producing mastitis in the district in which Savage's observations were made differ to a considerable degree from those which I have isolated. However, he brings out the point that most of the streptococci which he found in milk are of udder origin, and considers that the udder group is intermediate between the fecal and mastitis streptococci.

The group of low acid-producing hemolytic streptococci is interesting. Their hydrogen ion concentration readings are significant. According to Avery and Cullen's<sup>9</sup> observations, they fall well within the human group. In fact, in Table VIII of their paper the readings of three strains (M. 53, M. 86, and J. 1), isolated by the writer, are given. These strains were obtained from milk. At that time it was stated that they were not associated with mastitis, but might have been human contaminations, or arisen from the skin or feces of the cow. The latter postulation has been disproved, since hemolytic streptococci have not been found on the skin or in the feces of the cows supplying milk in this herd. These strains produce about the same amount of titratable acid in dextrose as the human streptococci. They differ from pathogenic human streptococci in character of the colonies produced on blood agar and in their ability to coagulate milk. They possess no virulence for mice and rabbits. It is possible, however, that such streptococci may be of human origin. Holman<sup>10</sup> designates streptococci which are hemolytic and ferment lactose but do not attack mannite, inulin, or salicin as *Streptococcus anginosus* (Andrewes and Horder). Several have reported the presence of such organisms in the human nose, throat, and tonsils. Perhaps these organisms gain access to the udder from the milker. At any rate they do not possess severe invasive characters for cattle, since their numbers are held in check in the udder. They differ from the non-

<sup>8</sup> Savage, W. G., *Rep. Med. Off., 1906-07, Local Gov. Bd., Appendix B, No. 4, 1908*, 3.

<sup>9</sup> Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.

<sup>10</sup> Holman, W. L., *J. Med. Research*, 1916, xxxiv, 377.

salicin-fermenting mastitis streptococci both in their hemolytic properties and in their inability to produce as much acid.

On morphological grounds they resemble diphtheroids, but have been placed with the streptococci for several reasons. In addition to forming chains in bouillon, they produce the characteristic hemolytic areas associated with hemolytic streptococci. Their fermentative characters resemble those of streptococci. In cultures that have grown on artificial media for considerable periods they produce in bouillon only chains of cocci.

Both the mastitis and the other group of streptococci have been observed in the market milk from this farm during the past 2 years. During this time diseases traceable to this milk supply have not been reported. This evidence points to the low pathogenicity of either group for consumers of milk. Either species may be eliminated as a source of severe epidemics of milk-borne sore throat.

#### SUMMARY.

The principal source of streptococci in milk is the cow's udder. The udder streptococci fall into two broad groups; those of the larger group agree in cultural characters and agglutination affinities with mastitis streptococci; the smaller group is composed of low acid-producing streptococci. The streptococci of the latter group produce clear zones of hemolysis about surface and deep colonies in horse blood agar plates. They attack dextrose, lactose, saccharose, and maltose, but do not ferment raffinose, inulin, mannite, or salicin. Acid production in dextrose by the members of this group is about the same as that produced by human streptococci under the same conditions. The limiting hydrogen ion concentration for these pleomorphic udder streptococci in dextrose serum bouillon is within the limits of the limiting hydrogen ion concentration observed by Avery and Cullen for human streptococci.

All the streptococci from the vagina, saliva, skin, and feces have been non-hemolytic. Those from the saliva form a heterogeneous aggregation in which individuals fermenting raffinose, inulin, and mannite predominate. From the skin a characteristic streptococcus has been found. It produces acid in dextrose, lactose, saccharose, mal-

tose, raffinose, mannite, and salicin, but fails to acidulate media containing inulin. The fecal streptococci are characterized by the formation of large amounts of acid in dextrose, lactose, saccharose, maltose, raffinose, inulin, and salicin. Mannite is not fermented. Neither the fecal nor the skin streptococci have been isolated from the bottled milk with any great frequency.



[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1920, Vol. xxxi,  
No. 5, pp. 633-645.]

## EPIDEMOIOLOGY OF BLACKHEAD IN TURKEYS UNDER APPROXIMATELY NATURAL CONDITIONS.

BY THEOBALD SMITH, M.D., AND H. W. GRAYBILL, D.V.M.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, February 24, 1920.)

The field experiments to be described in this paper are continuations of those made during the warm season of 1916 and published the following year.<sup>1</sup> The results of this earlier work indicated that the exposure of young incubator turkeys to older birds from infected flocks on infected ground produced disease within 2 weeks; exposure to older infected birds on fresh territory also produced disease, but after a somewhat longer period, and exposure to manifestly diseased young stock did not yield positive results. The last mentioned outcome might lead to various inferences, such as the elimination of unripe stages during active disease, the existence of an intermediate host, the need for a period of incubation in the soil, and so on.

The work of the years 1917, 1918, and 1919 was designed to repeat that of 1916, while furnishing ample material for the prosecution of other lines of work. However, as will be shown, the exposure of young turkeys to infected stock yielded cases of disease at irregular and uncertain intervals, so that intensive work on more specific problems had to be postponed until a method of producing blackhead with certainty and within a definite period of time could be worked out.

The experiments were conducted as heretofore with turkeys raised in the incubator and brooder and subsequently confined in outdoor enclosures of limited extent, varying in area from about 300 to 10,000 square feet. Each enclosure contained a small house in which the turkeys were kept at night. No attempt was made to cover these enclosures so as to exclude rodents, sparrows, and migrating birds.

<sup>1</sup> Smith, T., *J. Exp. Med.*, 1917, xxv, 405.

For it was considered of importance to determine if possible whether carriers, other than turkeys, exist. All experiments unless otherwise stated were conducted with turkeys raised in the incubator and brooder.

The term "blackhead" which has found its way into common use as referring to the disease due to the invasion of the walls of ceca and liver with a specific protozoan parasite, *Amœba meleagridis*, is used in these pages for convenience's sake.

### *Experiments of 1917.*

Three separate experiments were made in three outdoor enclosures. One was designed to test the infectiousness of young diseased turkeys, the other that of poultry from large flocks. The third contained controls. The turkeys used were hatched in three lots on dates beginning May 23 and ending June 16.

**Experiment 1.**—To test the infectiousness of young diseased turkeys, the latter were obtained from a nearby farm. Of a lot of eleven hatched by a hen and a turkey, three had died and the diagnosis had been confirmed on one by autopsy. Aug. 20. Four of the remainder, 5 to 6 weeks old, were introduced into the experimental enclosure containing four healthy turkeys. Aug. 25. One infected turkey died of blackhead. Aug. 26. A second died of the same disease. Aug. 29. The third died of coccidiosis. Blackhead lesions were not detected. Aug. 30. The fourth died of blackhead complicated with coccidiosis. The incubator turkeys were thus exposed for 10 days to both blackhead and coccidiosis in four living birds, and after the death of the latter to whatever soil infection resulted, until Nov. 9, a period of more than 2 months. No deaths occurred and no illness was observed. Nov. 9. One of the four was killed and found free from any lesions. The droppings of these birds had been examined in the course of the experiment and at the close the droppings of all were sedimented and examined for coccidia and ova but none were found. It should be stated that in the infected birds there were found in addition to the coccidia, specimens of *Heterakis papillosa*.

**Experiment 2.**—Aug. 20. To test the effect of exposure to chickens, four White Leghorns from four different flocks, three from New Jersey and one from Massachusetts, were placed in an outdoor enclosure and four turkeys placed with them. Turkey 175 was hatched on May 23, Nos. 178 and 185 on May 29, and No. 190 on June 16. No. 175 was first noticed to be sick on Sept. 28, 39 days after the beginning of the experiment. It was chloroformed and characteristic lesions of blackhead were found in ceca and liver. One individual of *Heterakis papillosa* was found in one cecum. The other three turkeys remained well. No. 185 was killed on Nov. 8, over 2½ months after the beginning of the exposure, and was

found free from any lesions or scars indicative of an attack. Four individuals of *Heterakis papillosa* were present. The two remaining turkeys were not killed but the feces were collected, washed, and sedimented. Neither ova nor coccidia were found.

*Experiment 3.*—Control enclosure. Eight incubator turkeys left after taking out those used in Experiments 1 and 2 were kept in an outdoor enclosure during the entire summer (Nos. 176, 179, 180, 182, 184, and 186 to 188). Aug. 25. No. 176 died. The feet of this turkey had been deformed and it was finally unable to walk. No blackhead lesions were present. Oocysts of coccidia were found in the ceca. No. 186 became sick on Sept. 14 and was killed on Sept. 21. Blackhead lesions were present in ceca and liver. No. 179 was killed Nov. 6 and No. 180 on Nov. 7, to determine if the birds had passed through a mild attack. No lesions or scars were present in either bird. Worms, ova, and coccidia were absent. The feces of all but No. 176 had been examined during the last week in Aug. and coccidia found in Nos. 184 and 187.

This group of experiments is significant from several points of view. It shows that blackhead may appear in an isolated group of turkeys and suggests that birds other than turkeys or hens may be carriers of the parasite. The disease appeared first in the control group on September 14 and 2 weeks later in the group with the chickens. It is highly probable that the same outside agencies brought the disease to both groups and that the chickens were not responsible. Singularly, the turkeys associated with three fatal cases of blackhead and one of coccidiosis remained healthy during the entire season, thus confirming earlier work that young diseased birds do not transmit blackhead. In accordance with this fact is the low morbidity in Experiments 2 and 3. Although each group was exposed to a case of blackhead occurring among its own numbers in September, yet no further illness occurred during the remainder of the season.

*Experiment 4.*—Towards the close of 1917 some individuals of the three groups (Experiments 1, 2, and 3) were brought together in the enclosure of Experiment 1 with a male, No. 125, which had been raised in the incubator in 1916 and exposed to disease during the summer of that year. This bird was therefore 1½ years old. A second group was formed on the grounds recently occupied in Experiment 3 as a control. Table I summarizes the condition when the groups were broken up on June 14, 1918. One in each group had been killed in Jan. and found normal. All in the control group were well. In the other group one died of blackhead on May 15 and two were ill about the same time, but recovered. They may have

passed through an attack of blackhead. One had been injured by the male and this may have accounted for the illness in one case. Both were still alive at the beginning of 1920. Several interpretations may be put on the outcome. Blackhead may have been transmitted by the old infected male (see Experiment 6), or by some individuals from Experiments 1, 2, and 3 which had become carriers, or by some outside agency.

TABLE I.

Exposed in enclosure of Experiment 1, Nov. 9, 1917, to Male 125, until June 14, 1918.

No. of turkey and group to which it had belonged.	Subsequent history.
181 (1)	June 14, 1918. Well.
189 (1)	Jan. 28, 1918. Killed. Normal. <i>Heterakis papillosa</i> in ceca.
184 (3)	May 23, 1918. Sick. Recovered.
188 (3)	" 15, 1918. Died. Blackhead.
178 (2)	" 20, 1918. Sick. Recovered.
Controls (in Enclosure 3).	
182 (3)	June 14, 1918. Well.
187 (3)	" 14, 1918. "
190 (2)	" 14, 1918. "
183 (1)	Jan. 30, 1918. Killed. Normal. <i>Heterakis papillosa</i> in ceca.

### *Experiments of 1918.*

**Experiment 5.**—This experiment was performed to test the relative infectiousness of older turkeys which had been exposed in the preceding year.

The flock to which young turkeys were to be exposed consisted of one from Experiment 1, two from Experiment 2, and three from Experiment 3 of 1917. With this flock the male referred to in the preceding experiment, No. 125, remained for a short time. They had occupied the same enclosure since the preceding year. The infectious character of the environment was demonstrated by the fatal case of blackhead of May 15, referred to in Experiment 4.

To imitate the usual conditions, one of these turkeys was made to incubate ten eggs. Six hatched on May 22. One young was killed in the nest, leaving five. One died on June 13, probably of general weakness. No infection could be demonstrated. On this day the remaining four were allowed to run with the older turkeys. All died of blackhead, one on July 22, one on Aug. 11, one on Aug. 13, and the last one on Sept. 8. After the death of the young stock, three additional young incubator turkeys were exposed on Sept. 17. Two of these contracted the disease on Oct. 2 and Nov. 12, respectively. The third, killed on

Nov. 25, 1919, showed some suspicious smooth areas in the ceca, but no definite lesions were found.

Two turkeys were introduced into this enclosure as late as Nov. 29. One of these contracted blackhead in Jan., 1919. The other remained well.

To test the susceptibility of chickens, four, hatched in an incubator and running only with incubator turkeys, were placed in this enclosure on Sept. 3. They

TABLE II.

Turkey No.	Source.	Exposure.	Result.
254	Hatched by No. 181 on May 22.	Exposed to mother turkey in coop until June 13, then to the old flock. " " "	July 22. Died of blackhead, after 39 days.
255	Hatched by No. 181 on May 22.	" " "	Aug. 11. Died of blackhead, after 59 days.
256	Hatched by No. 181 on May 22.	" " "	Aug. 13. Died of blackhead, after 61 days.
257	Hatched by No. 181 on May 22.	" " "	Sept. 8. Died of blackhead, after 87 days.
242	Hatched by No. 181 on May 22. Hatched by No. 181 on May 22.	" " "	June 13. Died, general weakness. Killed by accident in the nest.
264	Hatched in incubator.	Exposed on Sept. 17.	Oct. 5. Chloroformed. Affected with blackhead.
263	Hatched in incubator.	" " " 17.	Nov. 25, 1919. Killed. Normal except for a few possible scars in ceca.
262	Hatched in incubator.	" " " 17.	Nov. 19. Chloroformed. Affected with blackhead.
265	Hatched in incubator.	" " Nov. 29.	Jan. 21, 1919. Chloroformed. Affected with blackhead.
248	Hatched in incubator.	" " " 29.	Remained well.
Four chickens.	Hatched in incubator.	" " Sept. 3.	All remained well.

remained well and were killed, two 65 days and two 80 days after the beginning of the exposure. No lesions were found. Table II summarizes the results of this experiment.

Probably the most significant feature of this experiment is the fact that sooner or later nearly all the exposed died, but the period between the beginning of exposure and actual disease was very variable.

*Experiment 6.*—June 14. Four incubator turkeys were penned on new ground with the old male, No. 125, referred to in Experiments 4 and 5. July 9. Four additional young turkeys were placed in the same enclosure. Aug. 23. The old male was killed and found normal. Aug. 28. Experiment closed. None of the exposed showed signs of blackhead.

*Experiment 7.*—In this experiment young turkeys were penned with chickens from two sources. From each source a chicken had been found affected with blackhead. The first lot of chickens was penned with three healthy turkeys, beginning July 5. Dec. 20. Two of the turkeys were killed and found normal. The third was reserved.

Sept. 18. The second lot of chickens was penned with two turkeys. After an exposure of 69 days, one turkey was killed and found normal. The other was reserved for breeding.

TABLE III.

Lot No.	Date of hatching.	No. in each hatch.	Beginning of outdoor life.	Cases of blackhead.	No running together at the end of.
	1918		1918		
1	Apr. 24	10	May 9	None.	June, 48.
2	May 12	25	" 27	Nov. 27. (One.)	July, 39.
4	" 26	24	June 3	Dec. 20. (" )	Aug., 37.
5	" 28	(a) 10 (b) 8	" 1 " 3	June 22, Sept. 21, Nov. 6, 12, Dec. 14. (Five.)	Sept., 31. Oct., 30.
6	June 13	15	" 15	Oct. 26. (One.)	Nov., 15.

*Experiment 8.*—A study was made of the epidemiology of a large flock not exposed experimentally to disease. In the spring of 1918 the possibility that the eggs might be infertile led to the incubation of a relatively large number of eggs. The resulting hatch yielded a larger number of poult than could be taken care of adequately. The history of this group is therefore of interest from a practical standpoint. The various broods which went into the large flock are given in Table III.

The ground covered by the enclosure and the surrounding acres had not been used for poultry for several years and had been ploughed and planted in the spring. A small flock of incubator chickens was permitted to mingle with the turkeys to some extent. The entire group of turkeys was herded on an adjoining tract of several acres under supervision from time to time and then returned to the enclosure. When the vegetation in the latter had been destroyed the fence was moved along to cover fresher ground. The maximum number together at any time was 52, in the middle of June. Withdrawals and deaths from blackhead, diseases other than blackhead, and accidents, and the killing of eleven for food in Nov., gradually reduced the total number to fifteen at the end of Nov.

Among the miscellaneous early causes of death were crowding and chilling in early June, leading to twenty deaths. Four died following unthriftiness and emaciation, two of impaction of the small intestine, due to eating coarse food, three with congestion of lungs, one with inflammation of the ceca, and one with impaction of the kidneys with urates. These losses, thirty-one in all, occurred up to June 15, and might have been largely averted if the accommodations had been adequate to prevent overcrowding.

About the middle of June withdrawals began to be made for experimental purposes. Miscellaneous causes of death continued operative, although on a much smaller scale. One died with ceca inflamed, one from congestion of lungs, and one from an undefined cause, late in June. Two were lost from undefined causes and one from unthriftiness, in July. Among the infectious diseases, aspergillosis appeared early in July. Two birds died of this disease; two were chloroformed and the foci discovered at autopsy.

Blackhead appeared first in June. In all, eight cases were discovered. The distribution in time was quite irregular, as shown in Table III. Thus one case occurred in June, one in Sept., one in Oct., three in Nov., and two in Dec. The remaining twelve were killed in Dec. and no lesions found in any.

Ten from this same lot, which had been removed to other enclosures during the season, were killed late in Nov. and early in Dec., and all found free from traces of blackhead lesions.

### *Experiments of 1919.*

*Experiment 9.*—The fortuitous appearance of blackhead in enclosures protected in every way against the disease, except as it might be brought in by birds on the wing or small rodents, is well illustrated by the history of several flocks gradually merged into one larger flock during the summer of 1919.

The territory occupied during the season was an unused horse paddock enclosed by a high iron fence and not occupied by poultry for many years. The ground had been ploughed early in the spring and oats and grass sown. May 21, The first group of eight turkeys was moved to a brooder in the paddock when 9 days old. June 3. The brooder yard was enlarged to give more room. June 12. The brooder was replaced by a larger house. In the meantime one turkey had succumbed to blackhead on June 9. Others died on June 22, 29, July 2, 9, and 12. Two survived. To determine the infectious character of this flock and the soil, four additional young turkeys were introduced on June 30 (of which one died of softening of the bones soon afterward), three on Aug. 18; four on Sept. 5; and nineteen from another flock also on Sept. 5. In this last group, one had succumbed to blackhead on Aug. 13.

Sept. 5. The entire paddock was opened to the flock which now comprised thirty-one birds. Oct. 16. Twelve were taken out for a special feeding experiment. Oct. 20. One of the remaining nineteen died of blackhead. Nov. 17. Nine were taken out for another feeding test. Of the nine remaining, seven were

killed late in Dec. and all found free from lesions or scars of blackhead lesions. The ceca of all contained adult *Heterakis*, one bird carrying as many as twenty-nine specimens. The two left from the entire flock were penned with the older infected flock for breeding purposes. One of these died of acute blackhead on Jan. 30, 1920. Most of the data are brought together in Table IV.

The points of interest in the history of this flock are several. The unexpected, severe outbreak in June among birds still in the brooder resembles closely the occurrences so frequent upon farms where turkeys are raised. Thereafter only one other case occurred, making a mor-

TABLE IV.

Lot No.	Date of hatching.	No. of individuals	Introduced into enclosure.	Cases of blackhead	Remarks.
1	May 12	8	May 21	One died on June 9, 22, 29, July 2, 9, 12. (One dies of softening of bones.)	June 3. Yard enlarged.
1	" 12	4	June 30	One dies on Oct. 20.	
2	July 2	3	Aug. 18		
2 3	" 2 May 27	4 19	Sept. 5 " 5	None died. (One had died in this group on Aug. 13.)	Sept. 5. Entire paddock opened up to the thirty-one turkeys running together on Sept. 5.

tality of seven among thirty-eight birds. The other feature of interest is the absence of immunity among the survivors, although all had been exposed at one time or another to a case. Of six fed subsequently with infectious material, all died. One of the original lot, of which two survived out of eight, died over 6 months later of the acute disease, after having been penned with older infected turkeys for over a month.

**Experiment 10.**—June 18. In another enclosure placed on land ploughed up and seeded to oats and grass in the spring, a flock of twenty-three incubator turkeys, hatched on June 10, was placed. June 24. Six were taken out for experimental uses, leaving seventeen. July 13. A turkey died, possibly of coccidiosis,

as oocysts were abundant. Blackhead appeared in the middle of Aug. and cases occurred well into Nov., as shown in Table V.

Although only three out of ten died, yet the symptoms of the rest and the presence of suspicious smooth, often deeply pigmented areas in one or both ceca and of whitish scars or foci in the liver make the diagnosis of blackhead fairly certain. Possibly the first case might be eliminated. This interpretation would push the beginning of the epidemic into Sept.

After counting out the dead and recovered cases, there were left six which had not shown signs of disease. They were killed late in Nov. The organs were free from lesions or scars. *Heterakis* was abundant in all instances. The extent

TABLE V.

Turkey No.	Beginning of illness.	Result.	Further observations.
1919			
332	Aug. 19	Recovered.	Nov. 22. Killed. Normal.
354	Sept. 9	"	" 25. " Some scars in liver and smooth areas in ceca.
355	" 13	"	Reserved for breeding.
356	" 28	"	Jan. 21, 1920. Killed. Scars in liver and smooth and dark pigmented areas in ceca.
357	" 30	"	Nov. 24. Killed. Some whitish spots on liver and pigmented areas in one cecum.
358	" 30	Oct. 24. Died.	Blackhead.
359	Oct. 4	" 24. "	"
363	" 21	Nov. 13. "	"
364	Nov. 1	Recovered.	Nov. 22. Killed. Whitish foci in liver and pigmented areas in ceca.
365	" 6	"	Reserved.

of the disease may perhaps be accounted for by the fact that this flock was allowed to run over an adjoining part of the land on pleasant days, in the care of an attendant who drove them back into the smaller enclosure after 1 or 2 hours.

*Experiment 11.*—In this experiment a spontaneous outbreak among turkeys penned with incubator chickens occurred. The enclosure was placed on land which had been ploughed up and sown to grass and oats in the spring.

There were nineteen chickens in the flock. July 28. Two young turkeys were penned with them. Both became ill in 23 and 26 days respectively. One was chloroformed and the diagnosis of blackhead confirmed. Young stages of *Heterakis* were present. The other recovered and was killed in Nov. The liver showed healed foci and there were found a constriction of one cecum due to scar tissue and smooth areas in both ceca. Sept. 5. Three additional turkeys were placed in this group. All three contracted blackhead, one after 31 and the others after

34 days. One died. Mature *Heterakis* were found in the ceca. The others recovered. Both were killed in Jan., 1920, and in each there were changes indicative of healed blackhead lesions. The chickens remained clinically well. Eight were killed early in Oct. and two late in Nov. No lesions were detected. *Heterakis papillosa* was present in the ceca.

This group is of interest from the fact that all five turkeys placed with the chickens contracted blackhead. The relatively short period of incubation may have been due to the comparatively late exposure, since all experiments have pointed to an accumulation of disease-producing factors with the advance of the summer. The bearing of the chickens on the incidence of blackhead is not clear. It seems as if they may have picked up the virus with *Heterakis* and cultivated it with the latter in the ceca.

*Experiment 12.*—This experiment was designed to test the infectiousness of an enclosure recently occupied by a group of older turkeys as compared with the infectiousness of the same group on fresh soil.

(a) *Exposure to Older Turkeys on New Ground.*—The flock consisted of seven hens and a male, hatched during 1917 and 1918. June 2. They were cleansed and all soil was washed from the feet and they were then placed on grounds not heretofore used for poultry. The coops and nests were thoroughly scrubbed and cleansed before they were moved to the new grounds. June 3. Six young turkeys 7 days old were transferred in a brooder to the same new grounds and allowed to run out during warm weather. During cold or rainy days they were kept in the large coops occupied by the older turkeys at night to increase opportunities for infection. June 16. They were permitted to run freely with the older birds. In the meantime one had been smothered by the others. The remaining five did well up to Aug. 6, 64 days after the beginning of the exposure, when one became sick. One became sick on Aug. 7, one on Aug. 10, and two others on Aug. 15. One was killed on Aug. 20 and the diagnosis of blackhead confirmed. Some young and nearly adult *Heterakis* were found in the ceca, but no coccidia. The four remaining turkeys gradually recovered and all were well by the end of Aug. Evidence that they had passed through an attack was furnished when they were killed, two late in Dec. and two towards the end of Jan., 1920. Constrictions, obliterations of the longitudinal folds and pigmentation in the ceca, and scars or grayish foci in the liver were present.

In the group of twenty-two turkeys from which the above were taken one case occurred on Aug. 4. On Sept. 5 this group was merged with another group and one other case occurred in the combined flocks during the remainder of the season.

Aug. 23. The experiment was continued by placing three fresh turkeys into the enclosure with the older turkeys. The concentration of infectious material which had been going on was shown by the rapidity with which this new lot be-

came affected. One was ill on Sept. 4 and died on Sept. 12. The second was ill on Sept. 6 and it was killed on Sept. 9 and found diseased. Individuals of *Heterakis* were present in an immature stage in both cases. The third bird showed signs of illness on Nov. 17, but recovered. When killed on Jan. 22, 1920, the only indication of former disease was a large white healed focus in the liver. *Heterakis papillosa* was present.

(b) *Exposure on Grounds Vacated by Older Turkeys*.—The grounds occupied by the older flock during the winter were cleared, as stated under (a), on June 2, and remained vacant for 22 days. June 24. Six turkeys 2 weeks old were placed in it. 30 days later a young turkey showed signs of drooping and died of blackhead 15 days later. Three others contracted the disease, two on July 29 and one on Aug. 5. In all, both ceca were diseased. In two a few coccidia were found and in two larval nematodes. The fifth became sick on Sept. 27 and recovered. When killed late in Jan., 1920, there were a number of large scars in the liver and a smooth patch in each cecum. Nov. 2. The last of the six, an exceptionally vigorous bird, showed signs of disease and died 3 days later of blackhead.

Meanwhile, the loss of the birds was partly made good by introducing four young healthy turkeys on Aug. 18, when they were 47 days old. These also contracted blackhead. One died on Sept. 25, one on Oct. 20, and one on Oct. 21. The fourth showed signs of disease on Oct. 4, but recovered. It was killed on Jan. 22, 1920. The liver showed a few whitish foci and each cecum a smooth area on the mucosa.

In this experiment, the intensity of the infectious agent seems to have been nearly equal in the two enclosures. Disease appeared 30 days after the beginning of the exposure, in the vacated enclosure, and 64 days in the new enclosure containing the older birds. There is evidence that the infection became, if anything, more rather than less concentrated as the season progressed. This was indicated by the shorter time elapsing between exposure and disease.

The fact that 100 per cent of the exposed birds contracted the disease neutralizes the possible error involved in using birds from flocks in which spontaneous disease occurred. The percentage of such cases in the stock flocks was low and the disease first made its appearance some time after it had begun in the experimental enclosures.

One flock which consisted originally of twenty-three incubator turkeys, hatched on July 2, deserves mention here since blackhead did not appear in the enclosure during the greater part of the summer season. They were placed on a plot of ground on July 4, and allowed to run in a small yard on July 8 and in a larger enclosure on July 23. The flock was gradually reduced by withdrawals, four on

August 7, four on August 18, and three on August 23. Some died of non-infectious troubles. Seven were still present on September 5. These were later used in other experiments. They had thus been on the soil 59 days without any symptoms of blackhead.

During October a mild form of chicken-pox appeared in all but one of the groups of turkeys. It was first noticed among the turkeys in Experiment 9, and later in Experiments 12, *b*, 10, and 12, *a*. The mode of introduction remains unknown. This epizootic had no appreciable influence on the health of the birds.

#### SUMMARY.

The foregoing experiments in outdoor, unprotected enclosures demonstrate the difficulties surrounding the rearing of turkeys. These are discussed from another view-point<sup>2</sup> and to avoid repetition only a few outlying facts should be considered here.

The occasional presence of coccidia, the presence of *Heterakis papillosa* in the ceca, the occurrence of cases of aspergillosis and of chicken-pox in incubator-bred birds which did not come in contact with other domesticated birds, except in a few cases with incubator-bred chickens, show clearly that turkeys are picking up from the ground material deposited by other birds. The agent of blackhead must come from the same sources.

The field experiments show a steadily increasing concentration of the infection from 1917 to 1919, even though the ground had been ploughed and seeded before use. As a result, the various groups of turkeys became infected to a greater degree. The growth in the intensity of the disease may be in part ascribed to an accumulation on the soil of infectious agents during any given season after they had been introduced, but it is hardly acceptable as an explanation from season to season, when the soil was either virgin, as regards poultry yards, or ploughed deep and seeded before use. A more rational hypothesis is the gradual attraction of birds in larger numbers and greater variety on account of the food supply in the turkey enclosures and the more intensive cultivation of the land surrounding the laboratory and animal buildings since the beginning of the experiments in 1917.

<sup>2</sup> Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, **xxx**, 647.

The intensity of the outbreaks due to the confining of young turkeys with birds over a year old which had been infected during the preceding year, or on grounds previously occupied by them, was in all instances much greater than in the spontaneous outbreaks. The cases amounted to nearly 100 per cent of the exposed. On the other hand, the number of cases in the control flocks varied and was very low in some groups. It could have been kept down if the sick birds had been promptly removed and not permitted to recover on the same ground. However, the object of the experiment was not to suppress the disease, but to see to what extent it would develop.

It is self-evident that the results obtained apply strictly only to that part of the country where the experiments were made. We have at present no means of knowing whether the sources of infection would become more numerous and concentrated with a higher mean annual temperature, or the reverse. Only by using incubator turkeys exclusively for such tests and eliminating the older turkeys and domesticated birds as carriers, can the miscellaneous, at present not controllable sources of the agents of this disease in different localities and the chances of successful rearing be determined.



[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1920, Vol. xxxi,  
No. 5, pp. 647-655.]

## PRODUCTION OF FATAL BLACKHEAD IN TURKEYS BY FEEDING EMBRYONATED EGGS OF HETERAKIS PAPILLOSA.

By H. W. GRAYBILL, D.V.M., AND THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, February 24, 1920.)

Since the protozoan agent of blackhead was described and its relation to the lesions of the ceca and liver defined by one of the authors<sup>1</sup> in 1895, very little concerning the mode of transmission of the agent or the conditions favoring its invasion and multiplication has come to light. In 1895 Moore<sup>2</sup> fed the liver and ceca of three diseased turkeys to two healthy ones. The days of feeding were Nov. 28, Dec. 3, and Jan. 3. On Jan. 11, 44 days after the first feeding and 8 days after the last feeding, one of the turkeys died of blackhead. In another experiment beginning Nov. 28, Moore penned four healthy turkeys with two diseased ones. During the night for a period of about 2 weeks the feces of the sick ones were collected and fed, mixed with the feed, to the well ones on the following day. One of the two sick turkeys died in 3 days and the other survived. Of the four which received the discharges and were also exposed during the day to the sick ones and their environment, three became diseased. Two died in 22 and 27 days respectively; the third bird, well when killed 46 days after the beginning of the experiment, showed extensive lesions of blackhead.

In Sept., 1913, one of the authors<sup>3</sup> fed two incubator turkeys, hatched early in June of the same year and reared on virgin soil, the chopped up diseased ceca of two turkeys 2 to 3 months old. Both remained clinically well up to Dec. 24 when one was stolen. An autopsy on the other 3 days later showed it to be normal.

### *New Experiments in Feeding Feces.*

During the years 1918 and 1919 certain attempts to induce blackhead by feeding feces of older turkeys which had successfully passed through exposure to disease in the preceding year were made. These

<sup>1</sup> Smith, T., U. S. Dept. Agric., Bureau Animal Industry, Bull. 8, 1895, 7.

<sup>2</sup> Moore, V. A., U. S. Dept. Agric., Bureau Animal Industry, Circular 5, 1896, 1.

<sup>3</sup> Smith, T., J. Med. Research, 1915, xxxiii, 243.

attempts, although leading uniformly to negative results, are detailed below because of their bearing on subsequent more successful trials. It should be stated here that cohabitation in the same enclosure with the turkeys furnishing the feces produced blackhead in a certain number of young turkeys.

*Experiment 1.*—June 10, 1918. A male bird, 2 years old, was confined in an isolation unit after all soil had been washed from its feet. The feces were collected and fed to four young turkeys confined in a cage in the same unit. The turkeys were 30 days old. The feces were mixed with the food and fed on June 11, 12, 13, and 14. The experiment was closed on July 9 and the turkeys were released. At no time during a period of 28 days did they show symptoms of blackhead. These turkeys were used in other experiments during the season but none of them developed blackhead. One died on July 22 of a disease of the bones, resulting in all probability from confinement in the cage. The others were killed at the close of the year, one on Dec. 10 and the others on Dec. 20. The organs were uniformly normal.

*Experiment 2.*—Three turkeys, hatched on May 12, 1918, were fed feces of two old turkeys under the following conditions: The feces were collected on Sept. 2 and 3. A portion was fed on Sept. 2 with the food, morning, noon, and night. The remainder was placed in Petri dishes at room temperature until fed. The later feedings took place on Sept. 6, 9, and 13.

The young turkeys had been running with some chickens in a special enclosure and were kept in it except on the dates of the feeding. The feeding was done in a cage. They remained well and when two were killed late in Dec. no lesions were found. The older turkeys from which the feces were obtained belonged to an enclosure in which young had contracted blackhead. It will be noted that in this trial an incubation of the feces at room temperature for 3, 6, and 10 days respectively failed to make the test positive.

*Experiment 3.*—The feces of adult turkeys were fed to chickens.

In view of Milks<sup>4</sup> observations on the occurrence of blackhead in young chickens, it was believed that very young chickens would be suitable for feeding experiments, and such an experiment was undertaken in the spring of 1919, before young turkeys were available. This experiment was conducted in a brooder and was begun on Apr. 4, when the chicks were 8 days old. Six chicks were used, two of each of the following breeds: Rhode Island Red, White Leghorn, and Barred Plymouth Rock. The feces were collected as fresh as possible from the coops occupied by the old flock kept for breeding purposes. It consisted of eight birds, and in the main contained the same ones that were in it the previous year. Feces having different physical characters were collected. Those not fed fresh were kept in Petri dishes at room temperature. In all, from Apr. 4

<sup>4</sup> Milks, H. J., *Louisiana Agric. Exp. Station, Bull. 108*, 1908, 1.

to 17, feces were fed on 12 days. They ranged in age, counting from the date of collection, from less than 1 to 5 days. The chicks at no time showed symptoms of blackhead. One showed lameness due in all probability to confinement.

The manner of feeding the feces was then modified to reproduce more nearly natural conditions. On Apr. 17 a galvanized iron tray was placed in the outer compartment of the brooder and filled with sterilized soil. Feces were placed from time to time on the soil and the feed was always placed there. The soil was kept moist. Fresh and 1 day old feces were placed on the tray on Apr. 17 and on Apr. 18, 19, 20, 22, 23, 24, and 25 fresh feces were placed there.

Aside from lameness the chicks showed no other symptoms. Three of them were chloroformed on May 2, and three on May 6, 28 and 32 days respectively after the beginning of the experiment, and all were found free from blackhead.

After it had been found that by placing young turkeys with the old flock, subsequent to removal to new soil, they contracted blackhead, it was decided to undertake still another experiment feeding the feces of the old flock to young turkeys.

*Experiment 4.*—The turkeys of the old infected flock were removed by twos and placed in an indoor unit to facilitate collecting feces of all alike. The feces not fed fresh were kept in Petri dishes at room temperature. Beginning on Aug. 7, four young turkeys 36 days old, two white and two bronze, were fed in a cage. The feeding was carried out daily, with the exception of Sunday, to Aug. 19. After the feeding they were still kept indoors, but in a pen to enable them to be more active. The feces from eight older birds were used. When fed, they were either fresh or up to 8 days old. On Sept. 5 the young turkeys were removed to an outdoor enclosure. 23 days after the last feeding the experiment was closed. None had shown signs of disease in the meantime. Owing to a shortage of incubator turkeys these were used again in later experiments.

#### *Feeding Experiments with Embryonated Eggs of *Heterakis papillosa*.*

In experiments conducted in 1914,<sup>3</sup> 1916,<sup>4</sup> and since that time relating to the mode of transmission of the disease, it appeared that young diseased turkeys which did not recover were incapable of transmitting blackhead to healthy ones penned with them. In addition to this, it became evident that soil recently occupied by old turkeys was infectious to young ones.<sup>5</sup> These observations along with negative results in feeding feces suggested the existence of some

<sup>3</sup> Smith, T., *J. Exp. Med.*, 1917, xxv, 405.

<sup>4</sup> Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1920, xxxi, 633.

additional factor or factors in the transmission of blackhead. Previous experience and experiments gave no direct clue as to the nature of such a factor. There were a number of hypotheses which might fit the conditions observed or at least not be contradicted by them. The protozoan parasite invading the walls of the ceca and the liver evidently multiplied very rapidly when once started. It was also evident that this multiplication came to an abrupt stop and that the parasites in the tissues perished rapidly owing to an acquired immunity of the host or some other factor operating against the invading parasite at the height of the invasion. It was assumed that this parasite might be aided by some lesion or injury of the mucosa of the ceca to enter the tissues and multiply there, since the invasion in itself did not seem to be a normal part of the life cycle of the parasite. There was no evidence of the formation of any resistant stages in the tissues or of the normal discharge of the parasite outward, as, for instance, in coccidiosis. If the parasite was not to be regarded as wholly aberrant, whatever normal cycle there was seemed to belong to the lumina of the ceca. In harmony with this hypothesis a nematode parasite, *Heterakis papillosa*, occurring in the ceca of turkeys, chickens, and some other birds, was brought into the experiment as the possible associated factor.

*Experiment 5.*—This experiment consisted in feeding to young turkeys embryonated eggs of *Heterakis papillosa* and feces from adult turkeys. Worms were collected from the ceca of healthy chickens, killed for this purpose, by washing and sedimenting the contents and picking out the worms. These were cut up in a small quantity of normal salt solution to liberate the ova. The suspension was kept in Petri dishes at room temperature and distilled water added from time to time to make up for evaporation.

After the cultures had been kept for 17 days, examination showed that the ova contained living embryos. On Sept. 11 they were added to the feed of two turkeys which had been used in the preceding experiment. To ensure the complete ingestion of the mixture the turkeys were placed in a cage. On the same and the 2 following days feces from two older turkeys, collected on Sept. 10 and 11 were fed to the same birds. They were then placed with two other turkeys, also from the preceding experiment, which were to act as controls, in an outdoor enclosure.

Both infected turkeys became sick on Sept. 26; *i.e.*, 15 days after the feeding of ova. One died of blackhead after 3 days, the other after 6 days. The two control birds remained well and 43 days after the beginning of the experiment they were used in Experiment 7.

*Experiment 6.*—In this experiment the two factors, embryonated eggs of *Heterakis papillosa* and feces of adult turkeys, were kept distinct.

The young turkeys used in this experiment were from a group consisting of portions of three different broods of incubator turkeys, numbering in all about thirty individuals.<sup>6</sup> They had been running together for some time in a large enclosure. In some unknown way the parasite of blackhead had been introduced into this enclosure and six out of eight turkeys in one brood died of this disease during June and the first few days of July before the broods were mingled. Following these cases one turkey died of blackhead on Aug. 13 and another on Oct. 20. *Heterakis papillosa* was known to be present from some of the autopsies made. The latter was the only case that appeared in the remainder of the flock after the birds chosen for this experiment had been removed. Of the turkeys used Nos. 308, 309, and 312 were hatched on May 12, 1919, Nos. 360, 361, and 362 were hatched on May 27, and Nos. 345, 346, 350, 351, 352, and 353 on July 2. The turkeys were separated into four groups on Oct. 16: Nos. 309, 312, and 352 (controls) were fed nothing. Nos. 346, 350, and 360 received embryonated eggs plus turkey feces. Nos. 345, 353, and 361 received embryonated eggs only. Nos. 308, 351, and 362 received turkey feces only.

The *Heterakis* eggs used in this experiment were obtained from worms collected from the ceca of four chickens on Oct. 1, and were prepared for culture and incubated as in Experiment 5. The feces were obtained from two older turkeys of the infected flock, the same ones that were used in the preceding experiment, and were less than 48 hours old when fed.

The three feedings of ova and feces, of ova, and of feces, respectively, were conducted in separate enclosures and the three groups of turkeys were brought together after the feeding in a new enclosure with the controls, care being taken to remove all traces of soil from the feet by washing them thoroughly before the groups were finally assembled.

The result of the experiment is briefly told. Of the group which was fed eggs plus feces, Nos. 346 and 360 showed symptoms on Oct. 28, and No. 350 on Oct. 29; *i.e.*, in 12, 12, and 13 days respectively. They were killed and autopsied a few days later and the diagnosis was confirmed by the lesions in ceca and liver. Of the group fed ova only, Nos. 345 and 361 showed symptoms on Oct. 27, and No. 353 on Oct. 29; *i.e.*, in 11, 11, and 13 days respectively. Several days later they were killed and the autopsy showed marked lesions of ceca and liver.

The three that were fed feces only showed no symptoms of disease and were killed and autopsied, two on Nov. 10, and one on Nov. 11; *i.e.*, about 2 weeks after all those fed eggs had become sick. No lesions were found in these birds. A careful search for *Heterakis* in the cecal contents showed the presence of one mature male in No. 308, eight mature males in No. 362, and four females, two males, and one larval nematode in No. 351.

The three control birds showed no symptoms of disease and were killed on Nov. 10 and 11. One of these (No. 352) had lesions of blackhead; the others were entirely normal. In the ceca of No. 309, two mature males and one female, and in

No. 312, one female *Heterakis* were found. In the affected control both ceca showed many elevated indurations up to 0.5 cm. in diameter. The largest had a central superficial slough or scab. In the liver only a few small whitish foci, 1 to 2 mm. in diameter, and one focus of congestion were found. In fresh preparations from the ceca a few *Heterakis* eggs and one larval worm were found. In the washed and sedimented feces two mature and two immature females of *Heterakis* and many larval worms with attenuated posterior extremity and a bulbous esophagus were found. The mouth was without buccal capsule and provided with four papillæ.

The lesions of this case suggest that the disease may have been produced by something that occurred after the beginning of the experiment. This bird may have ingested ova from the adult worms in the other birds or perhaps embryonated eggs passing through the intestines of the others soon after they had been fed. In any case, the causes bringing about disease in this instance will probably be cleared up in future experiments when the external sources of the two factors, *Amœba meleagridis* and *Heterakis papillosa*, shall have been more definitely located.

*Experiment 7.*—This test may be regarded in a way as supplementary to Experiment 5. The two controls there surviving were fed 43 days after the beginning of Experiment 5 with embryonated eggs of *Heterakis papillosa*. The worms were collected from four chickens and the ova incubated as heretofore for 16 days, when they were fed mixed with the daily ration. The feeding was done on Oct. 24. One turkey became ill on Nov. 4, the other on Nov. 6; i.e., 11 and 13 days after feeding. They were chloroformed and the diagnosis was confirmed by finding lesions of ceca and liver.

*Experiment 8.*—Owing to the lateness of the season, only one additional test was undertaken. This consisted in feeding the contents of the ceca of turkeys affected with blackhead which had died or had been chloroformed and the feces of older turkeys, both incubated for 15 days, but free from *Heterakis* eggs.

The contents of the ceca of four diseased turkeys were suspended in normal salt solution and passed through a No. 40 wire mesh. The strained suspension was placed in Petri dishes in shallow layers. The incubation began on Nov. 3. Careful examination of the fluid under a low power failed to show the presence of ova.

Nov. 3. The feces of older healthy turkeys were collected, suspended in water, and passed successively through a tea strainer and wire screens of Nos. 40, 60, 100, and 200 mesh respectively. The final sediment was washed twice in normal salt solution and distributed into four Petri dishes. Nov. 14. The incubated dishes were carefully examined under a low power. Two *Trichosoma* ova but no others were detected.

Nov. 18. Nine turkeys, taken from the group from which those in Experiment 6 came, were selected. Three were reserved as controls, three fed cultures from diseased turkeys, and three cultures from older turkeys. After the feeding the three groups were brought together in one outdoor enclosure. No symptoms of disease appeared, and the birds were killed and autopsied as shown in Table I.

It will be noted that the turkeys were killed 21 and 29 days after the experiment had been begun. In the collection of the worms the contents of the ceca were washed and sedimented. Lesions were uniformly absent.

TABLE I.

Turkey No.	Date killed.	No. of <i>Heterakis</i> individuals.
1919		
310	Dec. 9	1 adult.
366	" 17	8 adults.
367	" 17	14 "
368	" 9	15 "
369	" 17	15 "
370	" 17	10 "
371	" 17	19 "
372	" 17	8 "
373	" 9	6 " and 2 young males.

## DISCUSSION AND SUMMARY.

In four experiments, three with young incubator turkeys and one with young incubator chickens, in which the feces of old turkeys from an infectious flock, kept at room temperature up to 5, 8, and 10 days, were fed, no infection resulted.

In an experiment in which two of four young incubator turkeys used in one of the above experiments were fed embryonated eggs of *Heterakis papillosa* and feces of turkeys from an infectious flock both contracted blackhead. Two controls remained well. Later they were fed embryonated eggs of *Heterakis papillosa* and both contracted blackhead.

In another experiment three incubator turkeys received embryonated eggs plus turkey feces from an infectious flock. All contracted blackhead. Three received embryonated eggs alone; all contracted blackhead. Three received turkey feces only; none contracted blackhead. Three controls received nothing; one showed blackhead lesions at the autopsy.

In a final experiment three turkeys were fed cultures of feces from the ceca of diseased turkeys, three were fed cultures of feces of old turkeys from an infected flock, and three controls were fed nothing. None contracted blackhead. The cultures of feces were prepared

precisely as were the earlier ones containing *Heterakis* eggs but without the latter.

From these experiments it becomes evident that blackhead may be produced in healthy incubator-raised turkeys, reared in the open in an environment where blackhead occurs, but out of direct contact with old turkeys and other poultry, by feeding cultures of embryonated eggs of *Heterakis papillosa*, prepared by cutting up the worms in isotonic salt solution and incubating the suspension at room temperature.

These very definite and clear-cut results outweigh any objections which may be raised against the use of turkeys which had been in earlier experiments and which came through such experiments without any signs of disease, or which came from control flocks in which spontaneous cases had occurred. The short time elapsing between feeding embryonated eggs and the first signs of disease made these experiments unusually impressive. It should be stated, furthermore, that from a precise individual record of all turkeys it was possible to select birds from control flocks in which the infection had either not appeared or was very low. All but two turkeys in flocks serving as sources of this material were killed at the close of the year. None at any time had shown symptoms of disease, and no scars or other abnormalities of ceca and liver were found. Furthermore, all other control birds and those in field experiments, with the exception of two reserved for breeding, were likewise killed. As a result of these autopsies, it was determined that of all birds in which symptoms of disease had not been recorded during life, none showed abnormalities or scars at autopsy. The protozoan factor in blackhead was probably disseminated when the first spontaneous cases occurred in the stock, unless it was present and made invasive by incubation in the cultures fed. This latter theory seems at present not acceptable because of the wholly negative outcome of Experiment 8.

The production of acute blackhead by feeding embryonated eggs to turkeys in whose ceca adults of *Heterakis papillosa* are already present seems incomprehensible at first thought. A tentative explanation to be offered is that the worms when invading the ceca in large numbers break down the resistance of the bird which is able to protect itself against a few. This may account for the very irregular

occurrence of cases in contact with older recovered birds on infected grounds. The rôle of *Heterakis* as a preliminary agent may also account for the continuing high mortality in turkeys in which the disease has been operating for so many generations to eliminate the most susceptible. It now seems highly probable that the turkey has become relatively resistant to the invasion of the protozoan parasite acting alone and that such invasion may require other agencies. Whether *Heterakis papillosa* is the only, or at any rate, the chief accessory agent or whether there are others, living or inert, which when ingested by the turkey assist in preparing the way for the destructive invasion of the walls of the ceca and the liver by *Amæba meleagridis* is a question now open to solution by experimentation.

The relation of common poultry to outbreaks of blackhead may be accounted for, at least in part, by the fact that they are hosts of *Heterakis papillosa*. How frequently they also carry *Amæba meleagridis* remains to be determined.

Since earlier communications have contained certain practical suggestions on the rearing of turkeys and the prevention of blackhead, it is not out of place here to point out that the additional information presented in this article simply emphasizes the suggestions already made. Turkeys should be raised in the incubator and brooder and kept away from older turkeys and poultry. The shelters should be moved from time to time to prevent a too concentrated infection of the soil with *Heterakis* ova. Inasmuch as the factors producing blackhead may be deposited by certain still undetermined birds on the wing, disease may be looked for at any time during the warm season. It is not, however, very readily transmitted, and in the experiments described elsewhere<sup>6</sup> the mortality from spontaneous blackhead was low. The flock should be looked over as frequently as possible, and whenever a turkey begins to droop, it should be isolated and killed if the drooping continues over several days. If such turkeys are allowed to recover, they should not be returned to the young flock but kept with older, presumably infected birds. Such birds are entirely satisfactory as a source of eggs, since there is no evidence that the latter transmit the infection.



# INDEX TO AUTHORS.

## A

- Allen, Frederick M. Experimental studies on diabetes. Series I. Production and control of diabetes in the dog. 1. Gross anatomic relations of the pancreas and diabetes, 409  
—. 2. Effects of carbohydrate diets, 427  
—. 3. Effects of protein diets, 449  
—. 4. Control of experimental diabetes by fasting and total dietary restriction, 469  
—. 5. Various failures of dietetic treatment, and their causes, 481

Amoss, Harold L. See FLEXNER and AMOSS, 45

Austin, J. Harold, and Van Slyke, Donald D. Determination of chlorides in whole blood, 321

## B

- Brown, J. Howard. The cultural differentiation of beta hemolytic streptococci of human and bovine origin, 503  
— and Orcutt, Marion L. Dairy infection with *Streptococcus epidemicus*, 517

Brown, Wade H., and Pearce, Louise. Experimental syphilis in the rabbit. I. Primary infection in the testicle, 133

## C

Courtney, Angelia M. See HOLT, COURTYNE, and FALES, 325, 347

- Cullen, Glenn E., and Van Slyke, Donald D. Determination of the fibrin, globulin, and albumin nitrogen of blood plasma, 397  
—. See LEVY and CULLEN, 313

## F

- Fales, Helen L. See HOLT, COURTYNE, and FALES, 325, 347

Flexner, Simon. Lethargic encephalitis. History, pathologic and clinical features, and epidemiology in brief, 119  
— and Amoss, Harold L. Experiments on the nasal route of infection in poliomyelitis, 45

## G

Gates, Frederick L. A method of standardizing bacterial suspensions, 35

Graybill, H. W., and Smith, Theobald. Production of fatal black-head in turkeys by feeding embryonated eggs of *Heterakis papillosa*, 577

—. See SMITH and GRAYBILL, 563

## H

Holt, L. Emmett, Courtney, Angelia M., and Fales, Helen L. Calcium metabolism of infants and young children, and the relation of calcium to fat excretion in the stools. Part I. Infants taking modifications of cow's milk, 325

—, —, and —. Part II. Children taking a mixed diet, 347

**J**

**Jones, F. S.** Source and significance of streptococci in market milk, 547

**K**

**Kligler, I. J.** See **OLITSKY** and **KLIGLER**, 19

**L**

**Larimore, Louise D.** See **Rous** and **LARIMORE**, 157

**Lawson, Mary R.** Crescentic bodies in aestivo-autumnal malaria; their migration and attachment to the surface of the red corpuscle, 91

**Levene, P. A.** Crystalline uridinophosphoric acid, 181

—. Properties of the nucleotides obtained from yeast nucleic acid, 189

—. The structure of yeast nucleic acid. V. Ammonia hydrolysis, 183

**Levy, Robert L.**, and **Cullen, Glenn E.** Deterioration of crystalline strophanthin in aqueous solution. Its relation to hydrogen ion concentration and a method for its prevention, 313

**Loeb, Jacques.** Influence of the concentration of electrolytes on some physical properties of colloids and of crystalloids, 219

—. Influence of a slight modification of the colloid membrane on the sign of the electrification of water, 201

—. Quantitative laws in regeneration. I, 243

—. The nature of the directive influence of gravity on the arrangement of organs in regeneration, 265

**Loeb, Jacques.** On the cause of the influence of ions on the rate of diffusion of water through colloid membranes. I, 279

**M**

**Murphy, James B.**, and **Nakahara, Waro.** The lymphocyte in natural and induced resistance to transplanted cancer. V. Histological study of the lymphoid tissue of mice with induced immunity to transplanted cancer, 1

—. See **NAKAHARA** and **MURPHY**, 13

**N**

**Nakahara, Waro**, and **Murphy, James B.** Studies on x-ray effects. V. Effect of small doses of x-rays of low penetration on the lymphoid tissue of mice, 13

—. See **MURPHY** and **NAKAHARA**, 1

**Noguchi, Hideyo.** Etiology of yellow fever. X. Comparative immunological studies on *Leptospira icteroides* and *Leptospira icterohaemorrhagiae*, 57

—. XI. Serum treatment of animals infected with *Leptospira icteroides*, 81

**Northrop, John H.** Concerning the hereditary adaptation of organisms to higher temperature, 259

—. A device for regulating the temperature of incubators either above or below room temperature, 255

**O**

**Olitsky, Peter K.**, and **Kligler, I. J.** Toxins and antitoxins of *Bacillus dysenteriae* Shiga, 19

<b>Oliver, Jean.</b> See Rous, WILSON, and OLIVER,	99	<b>Smith, Theobald, and Graybill, H. W.</b> Epidemiology of blackhead in turkeys under approximately natural conditions,	563
<b>Orcutt, Marion L.</b> See BROWN and ORCUTT,	517	—. See GRAYBILL and SMITH,	577
<b>P</b>			
<b>Palmer, Walter W.</b> See VAN SLYKE and PALMER,	377	<b>Stadie, William C.</b> A method for the determination of methemo- globin in blood,	307
<b>Pearce, Louise.</b> See BROWN and PEARCE,	133	— and Van Slyke, Donald D. Studies of acidosis. XV. Car- bon dioxide content and capacity in arterial and venous blood plasma,	301
<b>R</b>		<b>U</b>	
<b>Rous, Peyton, and Larimore, Lou- ise D.</b> Relation of the portal blood to liver maintenance. A demonstration of liver atrophy conditional on compensation,	157	<b>Uhlenhuth, Eduard.</b> Regeneration and neoteny,	113
—, Wilson, George W., and Oliver, Jean. Experiments on the pro- duction of specific antisera for infections of unknown cause. III. The effects of a serum pre- cipitin on animals of the species furnishing the precipitinogen,	99	<b>V</b>	
<b>S</b>		<b>Van Slyke, Donald D., and Palmer, Walter W.</b> Studies of acidosis. XVI. The titration of organic acids in urine,	377
<b>Smith, Theobald.</b> Mycosis of the bovine fetal membranes due to a mould of the genus <i>Mucor</i> ,	539	—. See AUSTIN and VAN SLYKE,	321
		—. See CULLEN and VAN SLYKE,	397
		—. See STADIE and VAN SLYKE,	301
<b>W</b>			
		<b>Wilson, George W.</b> See Rous, WILSON, and OLIVER,	99



# INDEX TO SUBJECTS.

## A

**Acid:**

- Organic acids, in urine, titration of (VAN SLYKE and PALMER) 377  
 Uridinphosphoric, crystalline (LEVENE) 181  
 Yeast nucleic, properties of nucleotides obtained from (LEVENE) 189  
 — —, structure of (LEVENE) 183

**Acidosis:**

- Studies (STADIE and VAN SLYKE) 301  
 — (VAN SLYKE and PALMER) 377

**Adaptation:**

- Hereditary, of organisms to higher temperature (NORTHROP) 259

**Aestivo-autumnal:**

- Malaria, crescentic bodies, attachment to surface of red corpuscle (LAWSON) 91  
 — — —, migration (LAWSON) 91

**Albumin nitrogen:**

- Blood plasma, determination in (CULLEN and VAN SLYKE) 397

**Ammonia:**

- Hydrolysis (LEVENE) 183

**Anatomy:**

- Pancreas and diabetes, gross anatomic relations (ALLEN) 409

**Antiserum:**

- Specific, for infections of unknown cause (ROUS, WILSON, and OLIVER) 99

## Antitoxin:

- Bacillus dysenteriae* Shiga (OLITSKY and KLIGLER) 19

**Aqueous:**

- Solution, crystalline strophanthin, deterioration (LEVY and CULLEN) 313  
 — — —, prevention of deterioration (LEVY and CULLEN) 313  
 — — —, relation of deterioration to hydrogen ion concentration (LEVY and CULLEN) 313

**Artery:**

- Blood plasma, arterial, carbon dioxide capacity in (STADIE and VAN SLYKE) 301  
 — — —, carbon dioxide content in (STADIE and VAN SLYKE) 301

**Atrophy:**

- Liver, conditional on compensation (ROUS and LARIMORE) 157

## B

**Bacillus:**

- dysenteriae* Shiga, antitoxins (OLITSKY and KLIGLER) 19  
 — — —, toxins (OLITSKY and KLIGLER) 19

**Bacteria:**

- Suspensions, standardization (GATES) .35

**Blackhead:**

- Turkey, epidemiology (SMITH and GRAYBILL) 563  
 — — —, production by feeding embryonated eggs of *Heterakis papillosa* (GRAYBILL and SMITH) 577

**Blood:**

- Corpuscle, red. *See* Erythrocyte.  
 Methemoglobin, method for determination of (STADIE) 307  
 Plasma, arterial, carbon dioxide capacity in (STADIE and VAN SLYKE) 301  
 —, —, carbon dioxide content in (STADIE and VAN SLYKE) 301  
 —, determination of albumin nitrogen of (CULLEN and VAN SLYKE) 397  
 —, — fibrin of (CULLEN and VAN SLYKE) 397  
 —, — globulin of (CULLEN and VAN SLYKE) 397  
 —, venous, carbon dioxide capacity in (STADIE and VAN SLYKE) 301  
 —, —, carbon dioxide content in (STADIE and VAN SLYKE) 301

- Portal, relation to liver maintenance (ROUS and LARIMORE) 157  
 Whole, determination of chlorides in (AUSTIN and VAN SLYKE) 321

**Body:**

- Crescentic, in aestivo-autumnal malaria, attachment to surface of red corpuscle (LAWSON) 91  
 —, — malaria, migration (LAWSON) 91

**C****Calcium:**

- Metabolism of infants and young children (HOLT, COURTNEY, and FALES) 325

**Cancer:**

- See* Carcinoma.

**Carbohydrate:**

- Diet, in diabetes (ALLEN) 427

**Carbon dioxide:**

- Arterial blood plasma, capacity in (STADIE and VAN SLYKE) 301  
 —, —, content in (STADIE and VAN SLYKE) 301  
 Venous blood plasma, capacity in (STADIE and VAN SLYKE) 301  
 —, —, content in (STADIE and VAN SLYKE) 301

**Carcinoma:**

- Transplantation, induced immunity, histology of lymphoid tissue (MURPHY and NAKAHARA) 1  
 —, lymphocyte in resistance (MURPHY and NAKAHARA) 1

**Cattle:**

- 'Mycosis of fetal membranes due to mould (SMITH) 539

- Streptococci, beta hemolytic, of human and bovine origin, cultural differentiation (BROWN) 503

**Chlorides:**

- Whole blood, determination of (AUSTIN and VAN SLYKE) 321

**Collodion:**

- Membranes, cause of influence of ions on rate of diffusion of water through (LOEB) 279

- , influence of slight modification of, on sign of electrification of water (LOEB) 201

**Colloid:**

- Influence of concentration of electrolytes on some physical properties of (LOEB) 219





<b>Compensation:</b>		<b>Deterioration—continued:</b>
Liver atrophy conditional on (Rous and LARIMORE)	157	Crystalline strophanthin in aqueous solution, prevention (LEVY and CULLEN) 313
<b>Concentration:</b>		<b>Determination:</b>
Electrolytes, influence on some physical properties of colloids (LOEB) 219		Blood plasma, albumin nitrogen of (CULLEN and VAN SLYKE) 397
—, — some physical properties of crystalloids (LOEB) 219		—, fibrin of (CULLEN and VAN SLYKE) 397
Hydrogen ion. <i>See</i> Hydrogen ion.		—, globulin of (CULLEN and VAN SLYKE) 397
<b>Corpuscle:</b>		Chlorides in whole blood (AUSTIN and VAN SLYKE) 321
Blood, red. <i>See</i> Erythrocyte.		Methemoglobin in blood, a method for (STADIE) 307
<b>Crescentic body:</b>		<b>Diabetes:</b>
<i>See</i> Body.		(ALLEN) 409, 427, 449, 469, 481
<b>Crystalline:</b>		Control, by fasting (ALLEN) 469
Strophanthin, deterioration, in aqueous solution, prevention (LEVY and CULLEN) 313		—, — total dietary restriction (ALLEN) 469
—, —, — aqueous solution, relation to hydrogen ion concentration (LEVY and CULLEN) 313		—, in dog (ALLEN) 409, 427, 449, 469, 481
<b>Crystalline uridinphosphoric acid:</b>		Pancreas and, gross anatomic relations (ALLEN) 409
<i>See</i> Acid.		Production, in dog (ALLEN) 409, 427, 449, 469, 481
<b>Crystalloid:</b>		<b>Diet:</b>
Influence of concentration of electrolytes on some physical properties of (LOEB) 219		Carbohydrate in diabetes (ALLEN) 427
<b>Culture:</b>		Diabetes, controlled by total dietary restriction (ALLEN) 469
Streptococci, beta hemolytic, of human and bovine origin, differentiation (BROWN) 503		—, dietetic treatment, causes of failures (ALLEN) 481
<b>D</b>		Mixed, calcium metabolism of children taking (HOLT, COURTNEY, and FALES) 347
<b>Dairy:</b>		—, relation to fat excretion in stools of children taking (HOLT, COURTNEY, and FALES) 347
<i>Streptococcus epidemicus</i> infection (BROWN and ORCUTT) 517		Protein, in diabetes (ALLEN) 449
<b>Deterioration:</b>		
Crystalline strophanthin in aqueous solution, hydrogen ion concentration, relation to (LEVY and CULLEN) 313		

<b>Diffusion:</b>	<b>Epidemiology—continued:</b>	
Rate, of water, through colloid membranes, cause of influence of ions (LOEB)	279	Encephalitis, lethargic (FLEXNER) 119
<b>Dioxide:</b>	<b>Erythrocyte:</b>	
Carbon. <i>See</i> Carbon dioxide.		Malaria, aestivo-autumnal, attachment of crescentic bodies to surface of (LAWSON) 91
<b>Dysenteriae:</b>	<b>Etiology:</b>	
<i>Bacillus</i> , Shiga, antitoxins (OLITSKY and KLIGLER)	19	Infections of unknown cause, specific antisera (ROUS, WILSON, and OLIVER) 99
—, —, toxins (OLITSKY and KLIGLER)	19	Yellow fever (NOGUCHI) 57, 81
<b>E</b>		
<b>Egg:</b>	<b>Excretion:</b>	
<i>Heterakis papillosa</i> , embryonated, blackhead in turkeys produced by feeding (GRAYBILL and SMITH) 577		Fat, in stools of infants and young children, relation of calcium to (HOLT, COURNEY, and FALES) 325, 347
<b>Electrification:</b>	<b>F</b>	
Water, influence of slight modification of collodion membrane on sign (LOEB) 201		
<b>Electrolyte:</b>	<b>Fasting:</b>	
Influence of concentration, on some physical properties of colloids (LOEB) 219		Diabetes controlled by (ALLEN) 469
—, —, on some physical properties of crystalloids (LOEB) 219		
<b>Embryo:</b>	<b>Fat:</b>	
<i>Heterakis papillosa</i> , embryonated eggs, blackhead in turkeys produced by feeding (GRAYBILL and SMITH) 577		Excretion in stools of infants and young children, relation of calcium to (HOLT, COURNEY, and FALES) 325, 347
<b>Encephalitis:</b>	<b>Feeding:</b>	
Lethargic (FLEXNER) 119		Blackhead in turkeys produced by feeding embryonated eggs of <i>Heterakis papillosa</i> (GRAYBILL and SMITH) 577
<b>Epidemicus:</b>	<b>Fetus:</b>	
<i>Streptococcus</i> , dairy infection (BROWN and ORCUTT) 517		Membranes, bovine, mycosis due to mould (SMITH) 539
<b>Epidemiology:</b>	<b>Fever:</b>	
Blackhead in turkeys (SMITH and GRAYBILL) 563		Yellow. <i>See</i> Yellow fever.

**G****Globulin:**

Blood plasma, determination of (CULLEN and VAN SLYKE) 397

**Gravity:**

Nature of directive influence of, on arrangement of organs in regeneration (LOEB) 265

**H****Hemolytic:**

Streptococci, beta, of human and bovine origin, cultural differentiation (BROWN) 503

**Heredity:**

Hereditary adaptation of organisms to higher temperature (NORTHROP) 259

**Heterakis:**

*papillosa*, embryonated eggs, blackhead in turkeys produced by feeding (GRAYBILL and SMITH) 577

**Histology:**

Tissue, lymphoid, in induced immunity to transplanted cancer (MURPHY and NAKAHARA) 1

**Hydrogen ion:**

Concentration, relation to deterioration of crystalline straphanthin in aqueous solution (LEVY and CULLEN) 313

**Hydrolysis:**

Ammonia (LEVENE) 183

**I****Icterohæmorrhagiae:**

*Leptospira*, and *Leptospira icteroides*, comparative immunological studies (NOGUCHI) 57

**Icteroides:**

*Leptospira*, and *Leptospira icterohæmorrhagiae*, comparative immunological studies (NOGUCHI) 57  
—, infection, serum treatment (NOGUCHI) 81

**Immunity:**

Carcinoma, transplanted, histology of lymphoid tissue in induced (MURPHY and NAKAHARA) 1

**Immunology:**

*Leptospira icteroides* and *Leptospira icterohæmorrhagiae*, comparison (NOGUCHI) 57

**Incubator:**

Temperature of, device for regulating, above room temperature (NORTHROP) 255

—, — for regulating, below room temperature (NORTHROP) 255

**Infection:**

Antisera, specific, for infections of unknown cause (ROUS, WILSON, and OLIVER) 99

*Leptospira icteroides*, serum treatment (NOGUCHI) 81

Poliomyelitis, nasal route (FLEXNER and AMOSS) 45

*Streptococcus epidemicus*, dairy (BROWN and ORCUTT) 517

Syphilis, primary, in testicle (BROWN and PEARCE) 133

**Ion:**

Cause of influence of, on rate of diffusion of water through collodion membranes (LOEB) 279

**L****Leptospira:**

*icterohæmorrhagiae* and *icteroides*, comparative immunological studies (NOGUCHI) 57

- Leptospira—continued:**
- icteroïdes* and *icterohæmorrhagiae*, comparative immunological studies (NOGUCHI) 57
  - , infection, serum treatment (NOGUCHI) 81
- Liver:**
- Atrophy, conditional on compensation (Rous and LARIMORE) 157
  - Blood, portal, relation to maintenance of liver (Rous and LARIMORE) 157
- Lymphocyte:**
- Carcinoma, transplanted, lymphocyte in resistance (MURPHY and NAKAHARA) 1
- Lymphoid tissue:**
- See* Tissue.
- M**
- Malaria:**
- Æstivo-autumnal, crescentic bodies, attachment to surface of red corpuscle (LAWSON) 91
  - , —, migration (LAWSON) 91
- Membrane:**
- Collodion, influence of slight modification of, on sign of electrification of water (LOEB) 201
  - , rate of diffusion of water through, cause of influence of ions (LOEB) 279
  - Fetal, bovine, mycosis due to mould (SMITH) 539
- Metabolism:**
- Calcium, of infants and young children (HOLT, COURTNEY, and FALES) 325, 347
- Methemoglobin:**
- Blood, method for determination of (STADIE) 307
- Method:**
- Bacterial suspensions, standardization (GATES) 35
  - Methemoglobin in blood, determination of (STADIE) 307
  - Strophanthin, crystalline, deterioration in aqueous solution, prevention (LEVY and CULLEN) 313
- Migration:**
- Malaria, æstivo-autumnal, migration of crescentic bodies (LAWSON) 91
- Milk:**
- Cow's, modifications of, calcium in stools of infants fed (HOLT, COURTNEY, and FALES) 325
  - Market, significance of streptococci (JONES) 547
  - , source of streptococci (JONES) 547
- Modification:**
- Cow's milk, infants taking modifications of (HOLT, COURTNEY, and FALES) 325
  - Slight, of collodion membrane, influence on sign of electrification of water (LOEB) 201
- Mould:**
- Mycosis of bovine fetal membranes due to (SMITH) 539
- Mucor:**
- Mycosis of bovine fetal membranes due to (SMITH) 539
- Mycosis:**
- Fetal membranes, bovine, due to mould (SMITH) 539
- N**
- Nasal:**
- Poliomyelitis, nasal route of infection (FLEXNER and AMOSS) 45

<b>Neoteny:</b>		<b>Plasma—continued:</b>
(UHLENHUTH)	113	Blood, determination of fibrin in (CULLEN and VAN SLYKE) 397
<b>Nitrogen:</b>		—, — globulin in (CULLEN and VAN SLYKE) 397
Albumin, in blood plasma, determination of (CULLEN and VAN SLYKE) 397		—, venous, carbon dioxide capacity in (STADIE and VAN SLYKE) 301
<b>Nucleic acid:</b>		—, —, carbon dioxide content in (STADIE and VAN SLYKE) 301
Yeast. <i>See Acid.</i>		
<b>Nucleotides:</b>		
Properties of, obtained from yeast nucleic acid (LEVENE) 189		
	O	
<b>Organ:</b>		<b>Poliomyelitis:</b>
Arrangement, in regeneration, nature of directive influence of gravity on (LOEB) 265		Infection by nasal route (FLEXNER and AMOSS) 45
<b>Organic acids:</b>		<b>Portal blood:</b>
<i>See Acid.</i>		<i>See Blood.</i>
<b>Organism:</b>		<b>Precipitin:</b>
Hereditary adaptation of, to higher temperature (NORTHROP) 259		Serum, effect on animals of species furnishing precipitinogen (ROUS, WILSON, and OLIVER) 99
	P	<b>Precipitinogen:</b>
<b>Pancreas:</b>		Serum precipitin, effects of, on animals of species furnishing (ROUS, WILSON, and OLIVER) 99
Diabetes and, gross anatomic relations (ALLEN) 409		<b>Protein:</b>
<b>Papillosa:</b>		Diet, in diabetes (ALLEN) 449
Heterakis, embryonated eggs, blackhead in turkeys produced by feeding (GRAYBILL and SMITH) 577		
<b>Pathology:</b>		R
Encephalitis, lethargic, pathologic features (FLEXNER) 119		<b>Red blood corpuscle:</b>
<b>Plasma:</b>		<i>See Erythrocyte.</i>
Blood, arterial, carbon dioxide capacity in (STADIE and VAN SLYKE) 301		<b>Regeneration:</b>
—, —, carbon dioxide content in (STADIE and VAN SLYKE) 301		(UHLENHUTH) 113
—, determination of albumin nitrogen in (CULLEN and VAN SLYKE) 397		Nature of directive influence of gravity on arrangement of organs in (LOEB) 265
		Quantitative laws (LOEB) 243
		<b>Resistance:</b>
		Carcinoma transplantation, lymphocyte in (MURPHY and NAKAHARA) 1

**Roentgen ray:**

Tissue, lymphoid, effect on, of small doses of low penetration (NAKAHARA and MURPHY) 13

**S****Serum:**

*Leptospira icteroides* infection, treatment (NOGUCHI) 81

Precipitin, effect on animals of species furnishing precipitinogen (ROUS, WILSON, and OLIVER) 99

**Solution:**

Aqueous, crystalline strophanthin, prevention of deterioration (LEVY and CULLEN) 313

—, —, relation of deterioration to hydrogen ion concentration (LEVY and CULLEN) 313

**Specificity:**

Antisera, specific, for infections of unknown cause (ROUS, WILSON, and OLIVER) 99

**Standardization:**

Bacterial suspensions (GATES) 35

**Streptococcus:**

*epidemicus*, dairy infection (BROWN and ORCUTT) 517

Hemolytic, beta, of human and bovine origin, cultural differentiation (BROWN) 503

Milk, market, significance (JONES) 547

—, —, source (JONES) 547

**Strophanthin:**

Crystalline, deterioration, in aqueous solution, prevention (LEVY and CULLEN) 313

**Strophanthin—continued:**

Crystalline, deterioration, in aqueous solution, relation to hydrogen ion concentration (LEVY and CULLEN)

313

**Suspension:**

Bacterial, standardization (GATES) 35

**Syphilis:**

(BROWN and PEARCE) 133

**T****Temperature:**

Higher, hereditary adaptation of organisms to (NORTHROP) 259

Incubators, device for regulating, above room temperature (NORTHROP) 255

—, —, regulating, below room temperature (NORTHROP) 255

Room, device for regulating temperature of incubators above (NORTHROP) 255

—, —, regulating temperature of incubators below (NORTHROP) 255

**Testicle:**

Syphilis infection, primary (BROWN and PEARCE) 133

**Tissue:**

Lymphoid, effect of small doses of x-ray of low penetration (NAKAHARA and MURPHY) 13

—, histology, in induced immunity to transplanted cancer (MURPHY and NAKAHARA) 1

**Titration:**

Acids, organic, in urine (VAN SLYKE and PALMER) 377

**Toxin:**

*Bacillus dysenteriae* Shiga  
(OLITSKY and KLIGLER)

19

**Transplantation:**

Carcinoma, induced immunity,  
histology of lymphoid tissue  
(MURPHY and NAKAHARA)

1

—, lymphocyte in resistance  
(MURPHY and NAKAHARA)

1

**Turkey:**

Blackhead, epidemiology  
(SMITH and GRAYBILL)

563

—, produced by feeding em-  
bryonated eggs of *Hetera-*  
*kis papillosa* (GRAYBILL and  
SMITH)

577

**U****Uridinphosphoric acid:**

Crystalline. See Acid.

**Urine:**

Organic acids, titration of (VAN  
SLYKE and PALMER)

377

**V****Vein:**

Blood plasma, venous, carbon  
dioxide capacity in (STADIE  
and VAN SLYKE)

301

— —, carbon dioxide con-  
tent in (STADIE and VAN  
SLYKE)

301

**W****Water:**

Diffusion, rate of, through col-  
loidion membranes, cause of  
influence of ions (LOEB)

279

Electrification, influence of  
slight modification of collo-  
dion membrane on sign  
(LOEB)

201

**X****X-ray:**

See Roentgen ray.

**Y****Yeast nucleic acid:**

See Acid.

**Yellow fever:**

Etiology (NOGUCHI)

57, 81



